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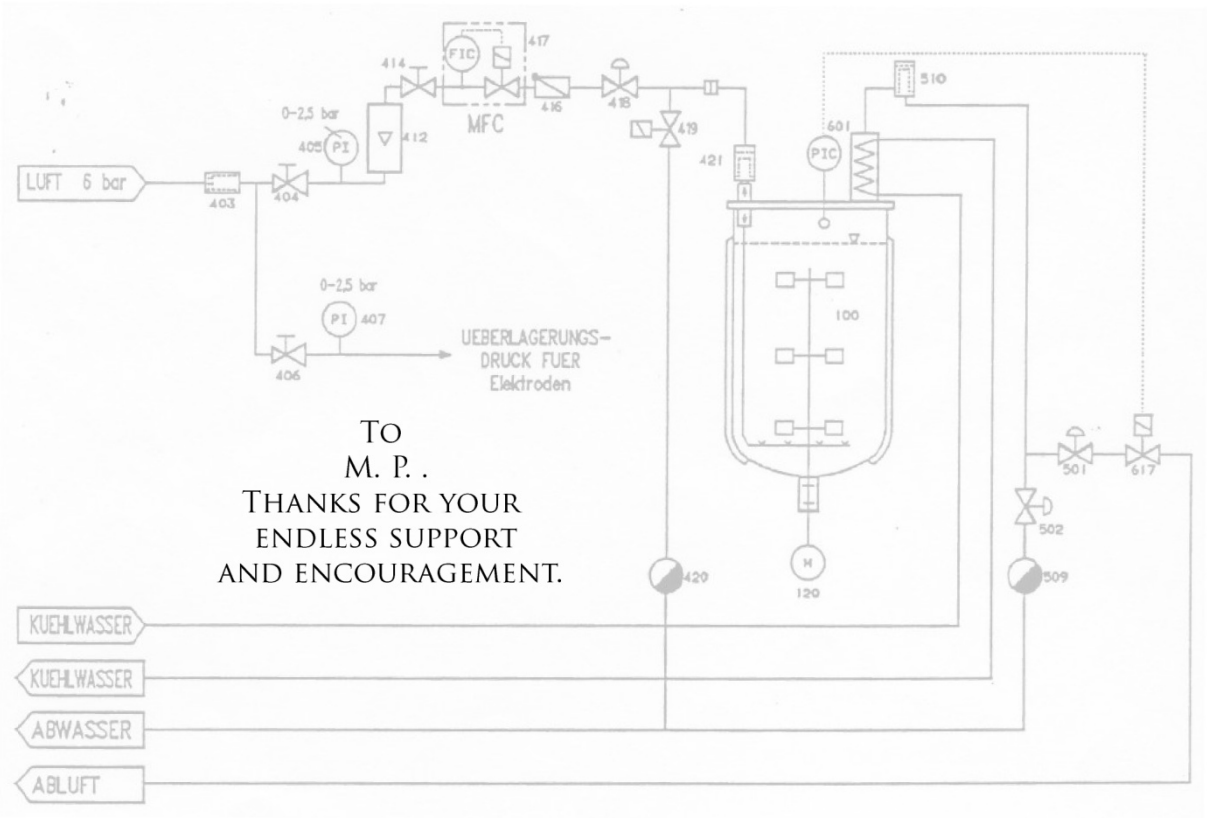

To
M. P. .
Thanks for your
endless support
and encouragement.

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Abbreviations

(C18 RP-)HPLC	(octadecyl carbon chain reverse phase) high performance liquid chromatography
ABC	ATP-binding cassette
Ac	acetyl
ActM	actin from *Microcystis aeruginosa* PCC 7806
APS	ammonium peroxodisulphate
ATP	adenosine triphosphate
bi	bicyclic
bp	base pair(s)
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTPs	desoxynucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
gDNA	genomic DNA
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	human immunodeficiency virus
IC_{50}	half maximal inhibitory concentration
IPTG	isopropyl ß-D-thiogalactoside
KDa	(kilo) dalton
LB	lysogeny broth
M. aeruginosa	*Microcystis aeruginosa*
MALDI-TOF MS	matrix-assisted laser desorption ionization–time of flight mass spectrometry

MALDI-TOF/TOF MS	matrix-assisted laser desorption ionization-tandem time of flight mass spectrometry
MCS	multiple cloning site
mdn (L, L1, L2, L3)	microviridin (L, L1, L2, L3)
mdnA/B/C/D/E	microviridin biosynthetic genes *A/B/C/D/E*
mdnJ	microviridin J
MndA/B/C/D/E	microviridin A/B/C/D/E protein
mono	monocyclic
MS	mass spectrometry
Mvn	microvirin
MWCO	molecular weight cut off
N298	Nies298
N843	Nies843
Nies298	*Microcystis aeruginosa* Nies298
Nies843	*Microcystis aeruginosa* Nies843
NRP	nonribosomal peptide
NRPS(s)	nonribosomal peptide synthetase(s)
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with Tween20
PCC	Pasteur Culture Collection
PCR(s)	polymerase chain reaction(s)
PIPES	1,4-piperazinediethanesulfonic acid
PK	polyketide
PKS(s)	polyketide synthase(s)
PMSF	phenylmethylsulfonylfluorid
POD	peroxidase from horseradish

PSD	post source decay
Qc	Quik change
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAG	sodium dodecyl sulfate polyacrylamide gel
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	Sec protein
SPR	surface plasmon resonance
TAE	Tris-acetate-EDTA
Tat	twin-arginin translocation
TBS-T	Tris buffered saline with Tween20
TE	Tris-EDTA
TEMED	tetramethylethylendiamin
TES	Tris-EDTA-saccharose
TFA	trifluoroacetic acid
tri	tricyclic
Tris	Tris(hydroxymethyl)aminomethane
TusA	sulfurtransferase TusA
UV/VIS	ultraviolet-visible
X-Gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

Nomenclature of microviridins

Nr.	name	crosslink	amino acid sequence	m/z, ion charge
1	mdnL	tri	Ac-YGGTFKYPSDWEDY	1737.7 $[M+Na]^+$
2	mdnL1	tri	Ac-GGTFKYPSDWEDY	1574.6 $[M+Na]^+$
3	mdn L2	tri	PSPEPTYGGTFKYPSDWEDY	2303.9 $[M+H]^+$
4	mdnL3	tri	IKSPSPEPTYGGTFKYPSDWEDY	2610.1 $[M+Na]^+$
5	mdnL1	bi	Ac-GGTFKYPSDWEDY	1570.7 $[M+H]^+$
6	mdnL	bi	Ac-YGGTFKYPSDWEDY	1733.7 $[M+H]^+$
7	mdnJ	tri	Ac-ISTRKYPSDWEEW	1684.7 $[M+H]^+$
8	mdnL3	bi	PSPEPTYGGTFKYPSDWEDY	2299.9 $[M+H]^+$
9	mdnL1 KYPAD	mono	Ac-GGTFKYPADWEDY	1572.7 $[M+H]^+$
10	mdnL TF→TL type	tri	Ac-YGGTLKYPSDWEDY	1703.7 $[M+Na]^+$
11	mdnL1 TF→TL type	tri	Ac-GGTLKYPSDWEDY	1540.6 $[M+Na]^+$
12	mdnL KWPSD type	tri	Ac-YGGTFKWPSDWEDY	1760.7 $[M+Na]^+$
13	mdnL1 KWPSD type	tri	Ac-GGTFKWPSDWEDY	1597.8 $[M+Na]^+$
14	mdnL KFPSD type	tri	Ac-YGGTFKFPSDWEDY	1721.7 $[M+Na]^+$
15	mdnL1 KFPSD type	tri	Ac-GGTFKFPSDWEDY	1558.9 $[M+Na]^+$
16	mdnL KYPSDS type	tri	Ac-YGGTFKYPSDSEDY	1616.5 $[M+H]^+$
17	mdnL1 KYPSDS type	tri	Ac-GGTFKYPSDSEDY	1453.3 $[M+H]^+$
18	mdnL field Y type	tri	Ac-YNVTLKYPSDWEEY	1794.9 $[M+H]^+$
19	mdnL1 field Y type	tri	Ac-NVTLKYPSDWEEY	1631.7 $[M+H]^+$
20	mdnL field F type	tri	Ac-YNVTLKYPSDWEEF	1778.8 $[M+H]^+$
21	mdnL1 field F type	tri	Ac-NVTLKYPSDWEEF	1615.7 $[M+H]^+$
22	mdnL PCC7822-2 type	bi	Ac-YQNTLKYPSDWEDY	1827.8 $[M+H]^+$
23	mdnL1 PCC7822-2 type	bi	Ac-QNTLKYPSDWEDY	1664.7 $[M+H]^+$
24	mdnL PCC7822-3 short type	bi	PIFTLKFPSDWEDS	1645.7 $[M+H]^+$
25	mdnL PCC7806-4 short type	bi	WTWKWPSDWEDS	1586.6 $[M+H]^+$

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Zusammenfassung

Microviridine sind cyanobakterielle, trizyklische Depsipeptide mit ungewöhnlich käfigartiger Struktur. Die Gene *mdnABCDE* kodieren für die ribosomale Biosynthese dieser Peptide. Zwei neuartige ATP-grasp Ligasen, MdnB und MdnC, katalysieren die Bildung von Lacton- und Lactamringen durch die Einführung von zwei ω-Ester-und einer sekundären ω-Amidbindung. Die Prozessierung wird von einer bislang unbekannten Peptidase durchgeführt. Neben den filamentösen *Nostoc* und *Planktothrix* gehört die einzellige, blütenbildende Cyanobakteriengattung *Microcystis* zu den Microviridin produzierenden Bakterien. Die potente inhibitorische Aktivität gegenüber Serinproteasen verleiht Microviridinen ökologische und pharmazeutische Relevanz.

Im Rahmen der vorliegenden Arbeit wurde eine kleine, stabile Microviridin Expressionsplattform konstruiert. Ein neuartiges Microviridin Gencluster aus *Microcystis aeruginosa* Nies843 wurde heterolog in *E. coli* exprimiert, bioinformatisch analysiert und mutiert. Das hochkonservierte PFFARFL-Motiv im Precursorpeptid MdnA wurde als Erkennungssequenz für die ATP-grasp Ligasen identifiziert. Manipulationen am C-Terminus des *leader*-Peptids führten zu einer Inhibierung der Aktivität von MdnB. Peptid-Protein-Interaktionen zwischen MdnA und den ATP-grasp Ligasen wurden untersucht. Es wurde gezeigt, dass der ABC-Transporter MdnE essentiell für die Zyklisierung und Prozessierung von Microviridinen ist, da er höchstwahrscheinlich einen Microviridin Biosynthesekomplex an der inneren Membran stabilisiert. Schließlich werden zwei mögliche Modelle für die Peptiderkennung und Prozessierung im putativen Biosynthesekomplex vorgeschlagen.

Punktmutationen in der Microviridin *core*-Sequenz offenbarten Flexibilität des Microviridin-Biosyntheseapparates für das *peptide engineering*. Es wurde eine Mutante konstruiert, deren inhibitorische Aktivität gegen Elastase um den Faktor >100 verbessert wurde. Durch die Konstruktion einer Precursoraustauschplattform konnten bisher kryptische Microviridine produziert werden. Diese Methode hat Potential für den Bau von Microviridinbibliotheken. Letztlich wird eine Hypothese zum Bindungsmechanismus von Microviridinen an Proteasen aufgestellt.

Abstract

Microviridins are the largest known cyanobacterial oligopeptides that are synthesized through a unique ribosomal route. These peptides comprise an unrivaled multicyclic cage-like structure, carrying two characteristic ω-ester and one ω–amide bond, which are introduced by the two novel ATP-grasp ligases MdnB and MdnC. In addition to the filamentous species *Nostoc* and *Planktothrix,* the unicellular, bloom-forming cyanobacterium *Microcystis aeruginosa* Nies843 is one of the microviridin producer strains. The potent serine protease inhibitory activity contributes to both ecological and pharmacological relevance of microviridins.

During this work, a small expression platform carrying the microviridin gene cassette *mdnABCDE* was established. The novel microviridin gene cluster was heterologously expressed in *E. coli* and analyzed using bioinformatics and mutational analysis. The strictly conserved PFFARFL motif in the precursor peptide MdnA was identified and characterized as a binding sequence for the ATP-grasp ligases. Manipulations of the C-terminal part of the leader peptide abolished lactam ring formation of microviridins. Protein interactions of MdnA with B and C were studied. The ABC-transporter MdnE was unveiled to be crucial for cyclization and processing of microviridins, probably holding and stabilizing a putative microviridin maturation complex at the inner membrane. Two initial models for the peptide recognition and processing have been proposed.

Point mutations in the microviridin core sequence showed some flexibility of the microviridin biosynthetic pathway to be used for peptide engineering. The exchange of a phenylalanine against a leucine in position 5 of the coding sequence resulted in more than a 100-fold increased inhibitory activity against the attractive drug target elastase. The possibility to express cryptic microviridin precursor peptides in a precursor exchange platform showed the potential to create peptide libraries. Finally, a hypothesis about the binding mechanism of microviridins is presented.

1 Introduction

1.1 Background about cyanobacteria

Cyanobacteria are a group of Gram-negative cosmopolitan prokaryotes. Findings of microfossils, stromatolites and chemical biomarkers in Australia and South Africa indicated cyanobacterial appearance about 2.8 billion years ago [Olson, 2006]. Belonging to the ancient colonizers of the earth, they played a key role as important primary producers of the oxygenic environment and their ability to nitrogen fixation further increased the biological capacity of the oceans and soils [Herrero and Flores, 2008].

Historically termed as blue-green algae, due to the prominent aquatic living space and the bluish-green color caused by green chlorophyll and the blue pigment phycocyanin, cyanobacteria were first classified botanically before they were proposed to be placed under the rules of the Bacteriological Code [Stanier, et al., 1978].

Cyanobacteria exhibit an unusual wide range of morphologies, including single-cell, colonial and filamentous forms. Cells differ in size from 0.5-60 µm diameter and thus are among the largest-sized cells known from prokaryotes, easily visible with the naked eye [Madigan and Brock, 2000]. Some species appear in balls (*Nostoc sp.*), other are able to form mats (*Oscillatoria*) (Figure 1-1) or blooms (*Microcystis, Anabaena*) (Figure 1-2).

Another feature of some planktonic cyanobacteria is the presence of gas vesicles that afford buoyancy and thus enable the bacteria to flow up and down to reach zones to conduct photosynthesis and find best nutrition [Walsby, 1994].

Cyanobacteria can colonize almost all habitats on earth. These adaptable microorganisms can conquer ecological niches that range from hot springs [Ionescu, et al., 2010], deserts [Lacap, et al., 2011], Antarctic ponds [Quesada and Vincent, 1997], rocks and soil [Rehakova, et al., 2011] to fresh and brackish water [Harke, et al., ; Lehtimaki, et al., 2000]. Forms of endosymbiosis with plants, protists and lichens [Dworkin, et al., 2006: ; Kneip, et al., 2008] were reported.

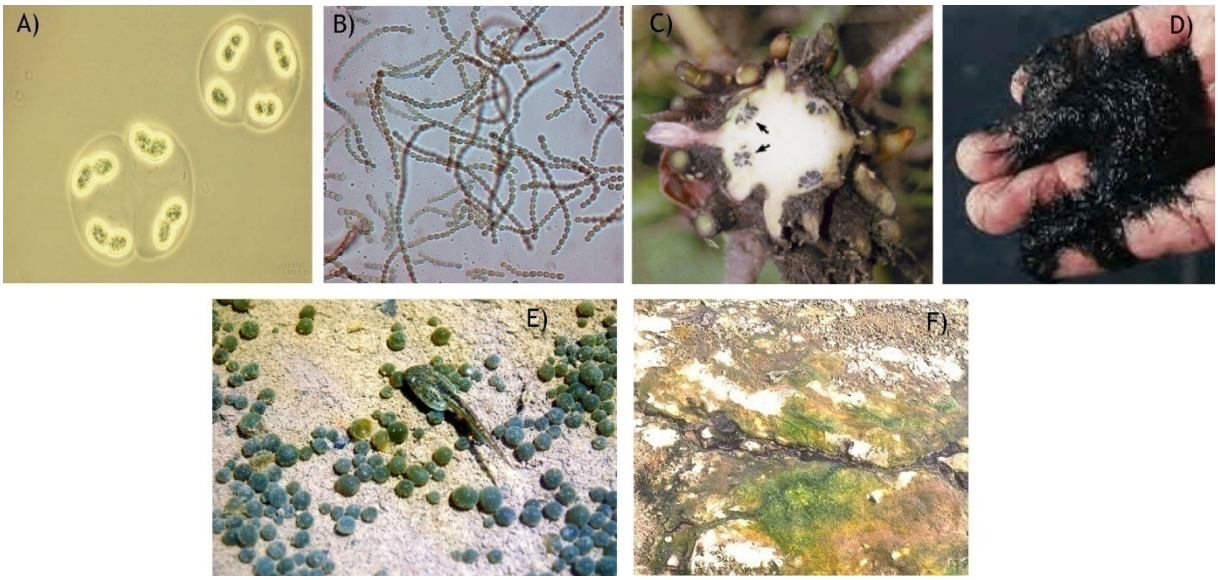

Figure 1-1: Diverse appearances of cyanobacteria

A) unicellular *Gloeothece sp.*, B) filaments of *Nostoc sp.* PCC7120, C) cross section through the stem of *Gunnera* with arrows indicating pockets of *Nostoc punctiforme*, D) *Oscillatoria* mat formation, E) free-living ball-like colonies of *Nostoc* in a fresh water pond in southern Oregon with a size of about 0.5 to 1.0 cm in diameter, F) a microbial mat in a hot spring in Oregon, containing cyanobacteria; picture sources are given in the appendix

1.1.1 Cyanobacterial metabolites

As a consequence of living in a variety of environments, cyanobacteria exhibit a broad spectrum of secondary metabolites with a wide range of useful characteristics, helpful for the producer to survive and fascinating for researchers and industry with regard to potential pharmaceutical, agricultural and biotechnological application. Major compounds that have been reported are the photoprotective scytonemins [Sorrels, et al., 2009], storage polymers as cyanophycin and poly-ß-hydroxybutyrate [Wu, et al., 2001: ; Ziegler, et al., 1998], several odourous metabolites [Watson, 2003], iron chelators as the dihydroxamate siderophore schizokinen [Goldman, et al., 1983: ; Murphy, et al., 1976], toxins and carcinogens like microcystin and cylindrospermopsin [Dittmann, et al., 1997: ; Falconer and Humpage, 2006], and compounds with cytotoxic [Ishida, et al., 2000], antibiotic [Schlegel, et al., 1998], antimalarial [Linington, et al., 2009], anti-HIV [Huskens, et al., 2010] and proteinase inhibiting activities [Ishitsuka, et al., 1990: ; Kodani, et al., 1998], some of them already in clinical phases. Although the great potential of these compounds is recognized for bioindustrial applications, the analysis, large scale production and especially the manipulation of these compounds often needs to be analyzed in heterologous hosts, as most cyanobacteria belong to the slow growing organisms that are frequently resistant to genetic manipulation, due to effective restriction barriers [Takahashi, et al., 1996].

1.1.1.1 Microcystis

Microcystis belongs to the unicellular, gas-vacuolated, toxic bloom-forming cyanobacteria, which have a coccoid cell shape and may aggregate to form colonies [Otsuka, et al., 2001].

The genus *Microcystis* produces a variety of secondary metabolites including the hepatotoxin microcystin [Tillett, et al., 2000], the chymotrypsin inhibitor cyanopeptolin [Bister, et al., 2004], the angiotensin-converting enzyme inhibitor microginin [Okino, et al., 1993], the cytotoxic microcyclamides [Ishida, et al., 2000] and the serine protease inhibitor microviridin [Ishitsuka, et al., 1990: ; Okino, et al., 1995]. These metabolites are synthesized via the ribosomal or nonribosomal route in *Microcystis*.

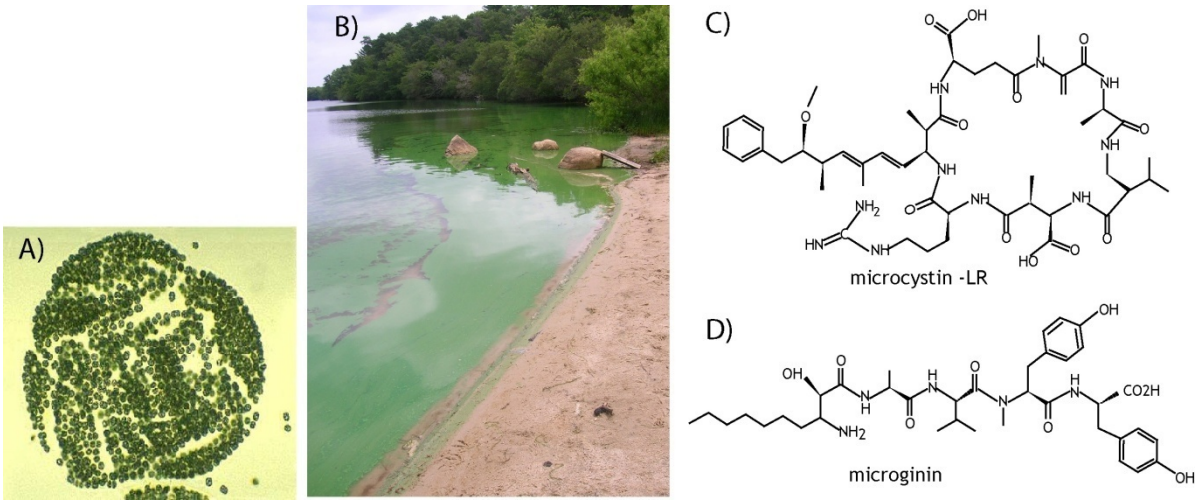

Figure 1-2: *Microcystis* and two representative compounds

A) *Microcystis* colony, B) bloom formation by *Microcystis,* C) structure of microcystin-LR, D) structure of microginin; picture sources are given in the appendix

1.2 The benefits of small cyclic compounds

The vast majority of bioactive cyanobacterial metabolites including microcystin, anabaenopeptin, cyanopeptolin and aeruginosin are products of either a multimodular nonribosomal peptide (NRP), polyketide (PK) or hybrid NRP-PK biosynthetic pathway [Dittmann, et al., 2001]. Over the past years the discovery of more and more ribosomally synthesized peptides, including the cyanobacterial microviridins and microcyclamides, has been seen [Velasquez and van der Donk, 2010]. In former times less celebrated as small-medicines for humans, compared to the nonribosomal peptides, the benefits including a stable cyclic composition, an attractive bioactive diversity, a short biosynthetic pathway and the possibility for peptide engineering of these scaffolds are just beginning to be appreciated [McIntosh, et al., 2009]. Mainly the cyclic composition of small ribosomally synthesized peptides confers to bioactivity, decreases the likelihood of proteolytic

degradation and improves thermal and chemical stability. Cyclic peptides have reduced conformational flexibility and therefore allow a greater preorganization of binding elements in comparison to linear molecules.

The advantages of ribosomal synthesis are obvious when compared to total synthesis of cyclic compounds. Total synthesis of cyclic natural peptides with interesting bioactivities and uniquely complex structures still presents formidable challenges. The processes are often time-consuming and expensive. Postranslational extremely modified compounds carrying intramolecular cyclizations occurring in nature are still difficult to be reconstructed. Extensive synthetic efforts are necessary to synthesize products with multiple chiral carbon centers and altering ring sizes [Marko, 2001]. As the product yields are often low, total synthesis is rarely a good alternative. In contrast, heterologous expression of ribosomal pathways in suitable hosts, leading to cyclic, highly modified bioactive peptides has been used in the study of several lantibiotics [Nagao, et al., 2007: ; Widdick, et al., 2003] and was shown to result in high product yields for microviridins [Ziemert, et al., 2010]. The commercial use of about 60 therapeutic peptides is reported and more than 150 are in clinical trials [Velasquez and van der Donk, 2010]. Ribosomal peptides are produced from a variety of living organisms, including eukaryotes, plants, fungi and archaea that afford similarities to the bacterial ribosomal peptides.

1.2.1 Peptides derived from ribosomal origin and their producers

The eucaryocins, peptide antibiotics produced by eukaryotes are typically small, thermostable, cationic peptides. They are often rich in cysteines and are able to form β-sheets or amphipathic α–helices [O'Connor and Shand, 2002]. Among the eukaryotic producers, poisonous mushrooms of the genera *Amanita*, *Galerina*, *Lepiota* and *Conocybe* produce bicyclic octa- and heptapeptides of 6-10 amino acids, referred to as amatoxins and phallotoxins. Both contain tryptophane-cysteine crossbridges, known as tryptathionine (Figure 1-3) [Walton, et al., 2010]. Cyclotides from plants of the genera *Violaceae* and *Rubiaceae* are head-to-tail macrocyclized mini-proteins of 28-37 amino acids with a cyclic cystine knot. They exhibit a wide spectrum of activities, ranging from antiinsecticidal, anti-HIV and cytotoxic to antimicrobial [Herrmann, et al., 2008]. Cone snails produce a repertoire of disulfite rich neurotoxins with potential pharmaceutical application. Ziconotide is the most prominent example, being on market to treat severe chronic pain [Garber, 2005]. Up to 140,000 other conopeptides last to be characterized [Lewis, et al., 2009]. Spiders and scorpions are expected to produce thousands of small useful peptides that have evolved for 400 million years [Bulaj, 2008].

Archaeocins, including the halocins and sulfolobicins, are peptide antibiotics and toxins produced from halophilic and hyperthermophilic archaebacteria. Halocins are hydrophobic compounds. They are noncationic and do not fold into α-helices, which indicates their mechanism of action is novel and potentially interesting for pharmaceutical effects. Only a few compounds as the small microhalocins S8 and HalR1 are characterized, but not exhaustively. Halocins exhibit a broad inhibitory spectrum against different haloarchaea, but not against bacteria. Sulfolobicin activity is also restricted to close related producers [O'Connor and Shand, 2002: ; Price and Shand, 2000].

Among the peptides produced from prokaryotes, the most intensively studied group includes the antimicrobial polypeptides called bacteriocins. They divide into lantibiotics, thioether amino acid and lanthionine-containing antibiotics, produced from Gram-positive and the microcins from Gram-negative bacteria. The best understood lantibiotic out of a group of 50 is nisin (Figure 1-3) from *Lactococcus lactis*, which has been used as a food preservative for more than 40 years now [Willey and van der Donk, 2007]. Moreover it is able to inhibit cell wall biosynthesis [Brotz, et al., 1998]. Lantibiotics as cinnamycin from *Streptomyces* strains show phospholipase-inhibiting characteristics and duramycin increases the chloride secretion in lung epithelium [Okesli, et al., 2011: ; Oliynyk, et al., 2010]. Carbacyclic labyrinthopeptins from *Actinomadura namibiensis* show antiviral activity and efficacy against neuropathic pain [Meindl, et al., 2010: ; Müller, et al., 2010]. The group of structural diverse microcins that mostly act as defensive peptides is secreted by enterobacteria and is rather small with about 15 representatives identified since their discovery in 1976 [Asensio and Perez-Diaz, 1976]. Some microcins, like the lasso peptide microcin J25 (Figure 1-3), contain only natural amino acids, others are extremely modified, as the DNA gyrase targeting linear microcin B17, being highly rich in glycine and containing eight pharmaceutically interesting heterocyclic oxazole and thiazole rings in the backbone [Jack and Jung, 2000]. A recent work examined the genetic potential for bacteriocin production in cyanobacteria and disclosed an enormous uncovered source [Wang, et al., 2011].

More than hundred unique small cyclic peptides composed of 7-20 amino acids that often contain oxazoles and thiazoles are known from the family of cyanobactins mainly produced by free living cyanobacteria, marine invertebrates sheltering symbiotic cyanobacteria and sponges [Donia, et al., 2008]. The best known representatives are the patellamides (Figure 1-3), being the first identified cyanobactins to be produced via the ribosome and thus leading to the inclusion into the ribosomally synthesized peptide group [Schmidt, et al., 2005]. Furthermore, trunkamides with potent antitumor activity [Salvatella, et al., 2003],

cytotoxic microcyclamides [Ziemert, et al., 2008] and others have been reported [Sivonen, et al., 2010].

Thiopeptides, containing nitrogen heterocycles, are known from several *Bacillus*, *Streptomyces* and *Frankia* strains. Thiocillin is the main representative. The enourmous number of 13 posttranslational modification reactions convert the thiocillin precursor molecule into the antibacterial antibiotic that inhibits protein biosynthesis [Wieland Brown, et al., 2009].

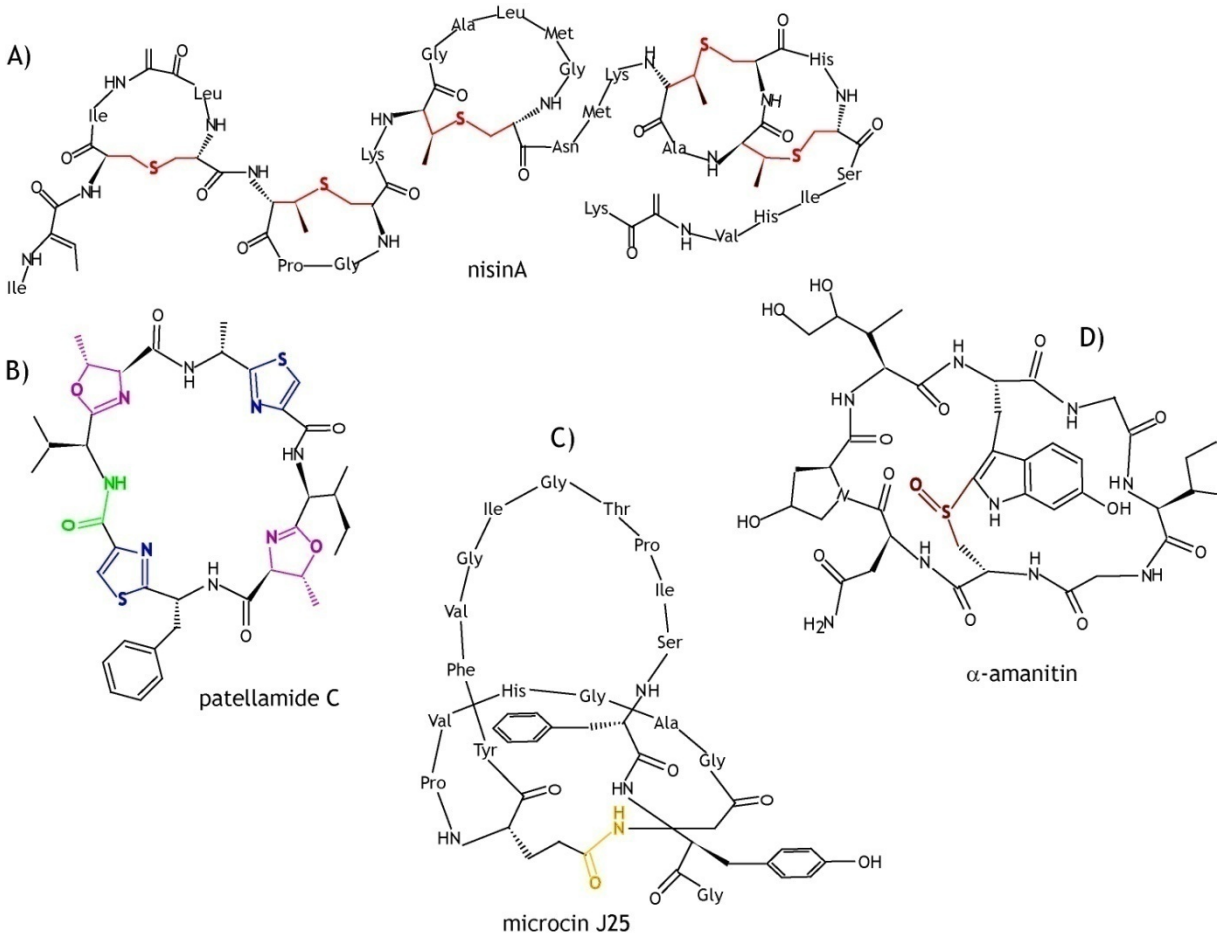

Figure 1-3: Representative structures from ribosomally synthesized peptides

Structures of cyclic bacterial ribosomally derived peptides are shown in A), B) and C), representing the lantibiotics (nisin A), the cyanobactins (patellamide C) and the microcins (microcin J25). A cyclic peptide produced by a eukaryotic mushroom is given in D) (α-amanitin). Postranlational modifications are highlighted with the following colors: (methyl)lanthionine bridges are shown in red; heterocyclization, as the formation of oxazoles, is given in purple; thiozoles are illustrated in blue; head to tail macrocyclizations are shown in orange and green; a cysteine-tryptophane crossbridge (trypthationin) is shown in claret-red.

The microviridins produced from cyanobacteria such as *Microcystis*, *Planktothrix* and *Nostoc* display an outstanding group among the ribosomally synthesized peptides. They comprise a unique cage-like tricyclic architecture that has been seen for no other

ribosomally synthesized candidate before. As microviridins are the central topic of this study, they will be explained in more detail starting with chapter 1.3.

1.2.2 Ribosomal peptide synthesis

The ribosomal peptide synthesis is restricted to the 20 proteinogenic amino acids. Ribosomal proteins so far reported share common features as they are synthesized from short precursor molecules, which are translated on the ribosome and extensively modified leading to remarkable structural diversity of the mature compounds. Bacterial precursor peptides often cluster with the genes involved in posttranslational modifications and secretion. At least one proteolytic step is needed to release a smaller active product. Leader peptides may carry recognition sequences to direct the modifying enzymes or to identify the proteolytic cleavage site (Figure 1-4). Ribosomal peptide synthesis is a relatively short pathway. A further major benefit is the clear link between the amino acid sequence and the final product. The exchange of only one amino acid or a few codons results in a modified compound providing opportunities to generate novel bioactive metabolites through rational pathway engineering [McIntosh, et al., 2009: ; Velasquez and van der Donk, 2010] (see also chapter 1.2.2.5).

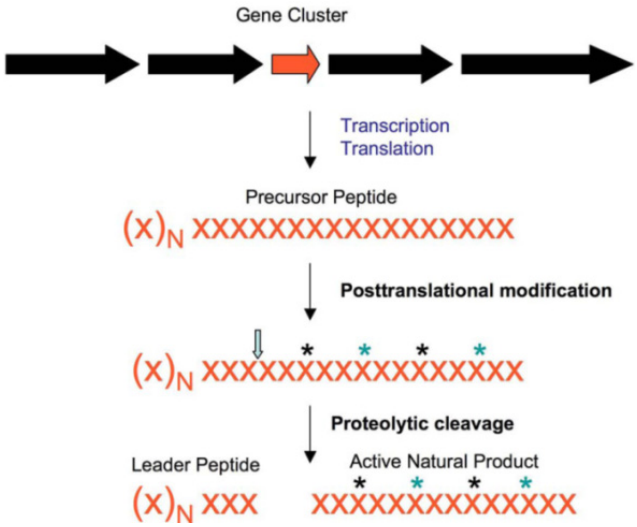

Figure 1-4: Schematic of ribosomal protein biosynthesis [McIntosh, et al., 2009]

A gene, encoding for a precursor molecule (orange) is located in the close vicinity of genes encoding for posttranslationally modifying enzymes (black). The precursor peptide is modified by these enzymes in both, the leader (blue arrow) and the core region (emphasized using asteriks). Ultimately the leader peptide is removed from the core by proteolytic cleavage.

1.2.2.1 Precursors

Some precursor peptides contain more than one core region plus flanking recognition sequences as seen for cyanobactins and amatoxins. Some eukaryotic precursor peptides, as those for conopeptides, contain a signal sequence at the N-terminus of the leader peptide

to direct it to the endoplasmic reticulum for further modifications [Oman and van der Donk, 2009].

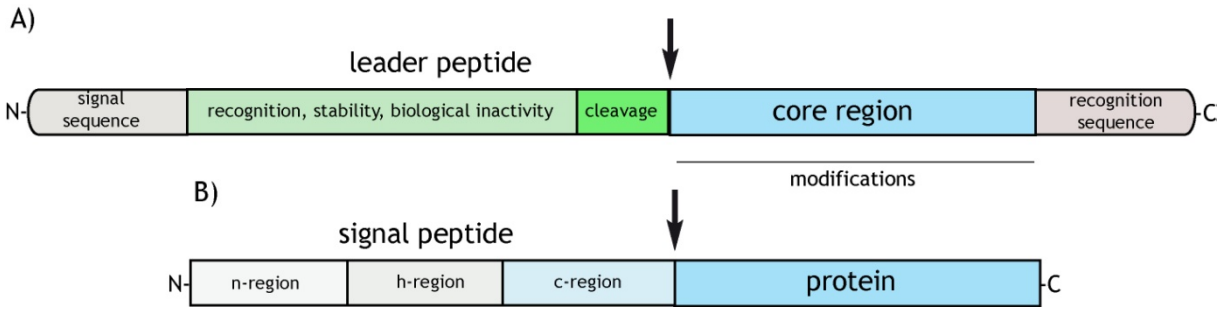

Figure 1-5: Schematic representation of leader and signal peptides

A) A typical precursor peptide of bacterial or eukaryotic origin is composed of a leader peptide and a core region. The leader peptide is assumed to be involved in different functions. N-terminal signal sequences, present in higher organisms, direct peptides to the endoplasmic reticulum. C-terminal recognition sequences are optionally. Core peptides are modified posttranslationally [Oman and van der Donk, 2009]. The typical composition of a standard signal peptide, used in combination with the Sec-dependent and Tat-pathway is represented in B). The signal peptide is divided into 3 regions, a rather polar n-region at the N-terminus, a hydrophobic h-region and the polar C-terminal c-region [Nothwehr and Gordon, 1989]. The protein to be translocated is attached to the signal peptide. Black arrows indicate cleavage sites in both peptides.

Several functions have been proposed for leader peptides. The most common is that of a secretion signal. Most leader peptides for natural product biosynthesis have humble similarity to the signal peptides from plants and microorganisms. They are necessary to initiate transport across the cytosolic membrane using the Sec-translocation pathway to transport unfolded proteins or the twin arginine pathway (Tat-pathway) for the translocation of folded secretory proteins across biological membranes. Standard signal peptides consist of an N-terminal rather polar region (n-region) with a positive net charge that is joined to the hydrophobic core region (h-region) comprising 2-15 amino acid residues, most essential for the targeting and membrane insertion. On the C-terminal side the hydrophobic core is flanked by a relatively polar region (c-region), which often contains helix-breaking residues as prolines or glycines and a signal that is recognized by a signal peptidase. Leader peptides may also carry recognition sequences for the posttranslational modification and/or export enzymes such as ABC-transporters. The double glycine or glycine-alanine motif of bacteriocins is well known as the recognition sequence for peptidase C39 domains of the cognate ABC- transporters [Dirix, et al., 2004: ; Michiels, et al., 2001]. Leader peptides may also participate in correct folding of the precursor. Furthermore, they are supposed to mediate stability against proteolytic degradation. Leader peptides may have a protective function for the producer cell. Some compounds gain their bioactivity once the leader is cleaved off, which usually happens during secretion [Lee, et al., 2006: ; Natale, et al., 2008: ; Oman and van der Donk, 2009: ; Pugsley, 1993]. This cooperation between the leader and the cognate processing and

export machinery is essential, as shown for nisin [Kuipers, et al., 2004]. *In vitro* studies demonstrated that microviridin K biosynthesis can only occur from a precursor peptide composed of a leader and a core sequence. A precursor peptide lacking the leader sequence was not a suitable substrate [Philmus, et al., 2008].

Until today comparatively little is known about the molecular secrets behind the recognition of the leader peptides by their modifying enzymes. A few recognition sequences were proposed for different bacteriocins, including nisin [van der Meer, et al., 1994], microcin J25 [Duquesne, et al., 2007] and lacticin 481 [Patton, et al., 2008].

1.2.2.2 Posttranslational modifying enzymes

As the ribosomal biosynthesis is restricted to the proteinogenic amino acids, which somehow limits the structural diversity, the myriad of characteristics is introduced through posttranslational modifications, leading to an enormous diversity of compounds compareable to the number of nonribosomally synthesized products. Modifications include reactions such as macro- and heterocyclization, lanthionine synthesis, prenylation, formylation, acylation and rather rarely occurring epimerization and glycosylation reactions. Proteolysis is the one modification almost all leader peptide derived ribosomal peptides have in common [McIntosh, et al., 2009]. Some enzymes are highly tolerant towards their substrate specificity, as shown for the cyanobacterium *Prochlorococcus* MIT9313. Up to 29 precursor molecules were transformed into a polycyclic peptide library, which emphasizes the extraordinary potential to create numerous new compounds by genetic engineering [Li, et al., 2010]. Similarly, the hypervariable PatE protein from symbiotic *Prochloron spp*. bacteria is tailored by a fixed set of enzymes [Donia, et al., 2006]

1.2.2.3 Bifunctional ABC-transporters and proteolytic cleavage recognition motifs

ATP-binding cassette transporters (ABC-transporters) are ubiquitous integral membrane proteins that use ATP-binding and hydrolysis to translocate a variety of different substrates, not only to acquire nutrients and excrete waste products across membranes, but also to fulfill a multitude of regulatory functions. ABC-transporters are mainly reported from prokaryotes, but eukaryotic examples are also known. In Gram-negative bacteria they transport proteins via the periplasm through the two membranes into the extracellular space. Some transporters need the help of specific substrate-binding proteins in the periplasm [Higgins, 1992].

ABC-transporters are often recognized in the close vicinity to a ribosomal peptide encoding gene cluster. They are minimally composed of 4 domains, 2 transmembrane-spanning transport channel forming domains and 2 cytosolic nucleotide-binding domains (Figure 1-6, A), which are designated through their characteristic Walker A ((GXXXXGKT/S) with X being any amino acid) and B motifs. These two cooperatively acting domains of about 150 amino acids are significantly involved in ATP-binding and hydrolysis [Rees, et al., 2009]. The loss of function of one domain results in a complete breakdown of transport function [Schneider, 2000]. However, the domains can be also fused into larger, multifunctional polypeptides.

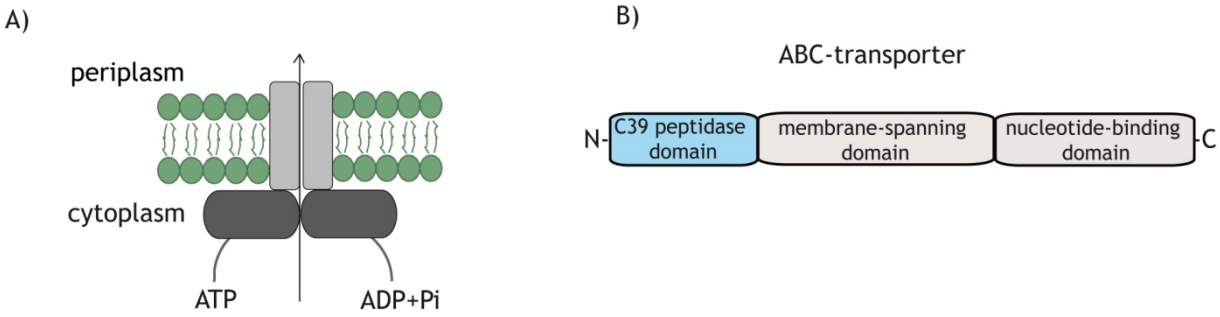

Figure 1-6: Simplified representation of an ABC-transporter

A) Modular composition of a bacterial ABC-transporter. The transporter consists of two transmembrane (light gray) and two nucleotide-binding domains (dark gray), which are located on the cytosolic side. The transport function depends on ATP hydrolysis. Assisting proteins in the periplasmic space, as mentioned in the text, are not shown. A schematic representation of an ABC-transport protein containing a C39 peptidase domain at the N-terminus (blue), followed by a membrane-spanning and a nucleotide-binding domain (gray) is shown in B).

Some of these transporters hold a bifunctional role. Beside the ATP-dependent transport they act as the maturation peptidase with an N-terminal C39 peptidase domain (Figure 1-6, B) that is missing from other ABC-transporters [Dirix, et al., 2004: ; Havarstein, et al., 1995]. During secretion, these transporters recognize a double glycine motif, the rather rarely occurring glycine-alanine or a glycine-serine motif in the corresponding leader peptides from group II lantibiotics and nonlantibiotic bacteriocins, to cleave and release the correctly processed active product [Havarstein, et al., 1994: ; Michiels, et al., 2001]. As not all ribosomal precursor peptides contain double glycine motifs, there are other options to liberate the mature compound. Specified enzymes are good candidates, as reported for the cyanobactins. A subtilisin-type protease PatA, encoded in the patellamide gene cluster, was shown to sequentially remove the two peptide cassettes present in cyanobactin precursor peptides [Schmidt, et al., 2005]. The protease starts from the C-terminus by probably using a recognition motif located in the recognition sequences (as shown in Figure 1-5). Cyclotides from plants are proposed to be cleaved similarly to the cyanobactins but by an asparagine peptidase [Saska, et al., 2007]. The core region of the amatoxin precursor peptide, encoding a mushroom poison from *Conocybe albipes*, is

cleaved N- and C-terminally by a proline oligopeptidase [Luo, et al., 2009]. Proline-specific peptidases can cover almost all situations were a proline is present and thus are recognized to be potential candidates for precursor processing [Cunningham and O'Connor, 1997]. Aberrantly proteases might be located in the chromosome of the producing bacteria and not in the respective gene cluster, as shown for microcin B17 [Duquesne, et al., 2007] and assumed for type III lantibiotics [Meindl, et al., 2010].

1.2.2.4 The formation of biosynthetic complexes

The formation of a biosynthetic complex, involving the posttranslational modifying enzymes, the transporter, scaffolding proteins and a precursor peptide has been observed to be essential for the biosynthesis of a number of ribosomally synthesized peptides. The nisin biosynthesis and immunity requires 11 clustered genes *nisABTCIPRKFEG*, whereupon 5 are necessary to form a membrane-associated multimeric complex involving NisABCTP, as shown in Figure 1-7 [van den Berg van Saparoea, et al., 2008].

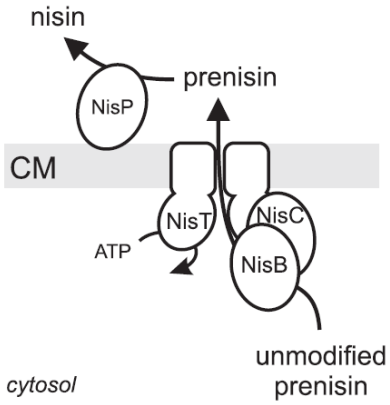

Figure 1-7: The formation of a membrane-associated biosynthetic complex using the example of nisin biosynthesis [van den Berg van Saparoea, et al., 2008]

The nisin precursor peptide (prenisin) is targeted to the cytoplasmic membrane (CM) associated biosynthesis complex consisting of a dehydratase NisB, a cyclase NisC and NisT, an ABC-type transporter. The mature nisin is secreted by NisT and cleaved ousite the cell by NisP, to release the active nisin.

Microcin B17 biosynthesis was the first biosynthetic pathway of a ribosomally synthesized peptide that has been reconstituted *in vitro*. At least 3 gene products are necessary to introduce oxazole and thiozole rings into the precursor peptide McbA *in vitro*. McbD is the prime recognition site for the substrate McbA that is correctly positioned through the polyglycine linker. McbB and McbC finally process microcin B17 by cyclization and dehydrogenation reactions, as shown in Figure 1-8 [Milne, et al., 1999].

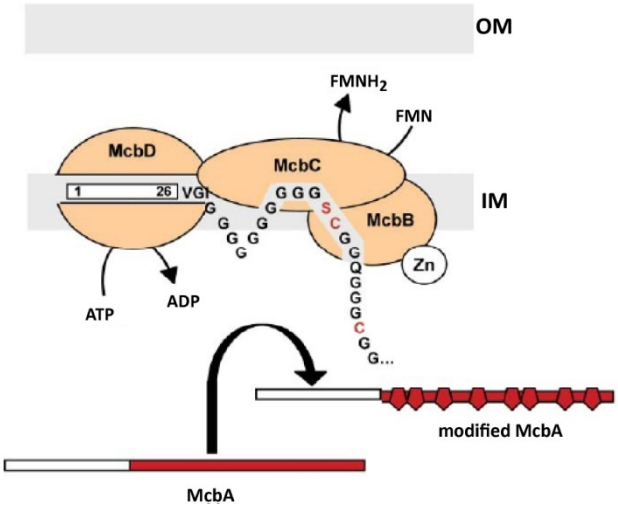

Figure 1-8: The formation of a membrane-associated biosynthetic complex using the example of microcin B17 biosynthesis. This figure is partially adopted from [Duquesne, et al., 2007].

The microcin B17 synthetase consists of 3 proteins. McbD is responsible for initial substrate recognition and precursor stabilization. The posttranslationally modifying enzymes McbB and McbC are responsible for cyclization and dehydration of the precursor peptide McbA. McbB is zink dependent. OM: outer membrane. IM: inner membrane. Zn: zink

1.2.2.5 Engineering of ribosomally synthesized peptides

Engineering gene-encoded peptides offers both, the possibility to unlock information surrounding structure-activity relationships and to generate new classes of compounds with improved bioactivities, stabilities, aquaeus solubilities and pharmakoginetics. Ribosomal synthesis permits methods to alter the structure of a peptide much easier compared to other natural products. In general, subtle changes in the core of different precursors, as mentioned below, showed once in a while a beneficial impact for the production of new and improved peptides, whereas broad and profound manipulations rather led to a knock-down of the complete biosynthetic pathway. Alterations to residues involved in cross-linking or bridge formation have been shown to result predominantly in a loss or at least a decrease of bioactivity and production [Cooper, et al., 2008: ; Philmus, et al., 2009].

The possibility to manipulate ribosomally synthesized products using *in vivo* mutagenesis has been demonstrated in several cases. The four contigious identical precursor genes *tclE-H* in the thiocillin gene cluster were exchanged against a single plasmid based copy of *tclE*. Thiocillin production was rescued. The introduction of a series of mutations into the thiocillin core peptide led to a number of variants, including two with 2-4-fold improved activity against methicillin-resistant *Staphylococcus aureus* [Acker, et al., 2009]. A variant generating system for the lantibiotic actagardine has been recently constructed. The deletion of the *garA* gene from *Actinoplanes garbadinensis* generated a mutant incapable of producing actagardine. Co-expression of a cassette of the actagardine encoding gene

garA from a plasmid restored the production. Although peptides with enhanced activity were so far not detected from the small screened library, the potential for the rapid construction of new variant is given [Boakes, et al., 2009]. Several other lantibiotics including the prototype lantibiotic nisin [Field, et al., 2008], nukacin ISK-1 [Islam, et al., 2009] and mersacidin [Appleyard, et al., 2009] have been successfully manipulated for increased bioactivity, which led to the assumption that the `Golden era´of lantibiotic bioengineering is entered [Field, et al., 2010].

To overcome problems of intolerance of the biosynthetic machinery to certain changes or toxicity to the producer due to novel bioactivities during *in vivo* expression, reconstitution and manipulation of biosynthetic pathways ultimately led to *in vitro* systems. *In vitro* mutasynthesis of lacticin 481 produced the most interesting mutants to date. Two lacticin 481 manipulated candidates with enhanced bioactivity contain nonproteinogenic amino acid substitutions [Levengood, et al., 2009], which is an anomaly that is impossible to be obtained using *in vivo* methodologies. An *in vitro* investigation into the mechanism of the cross-linking enzymes in microviridin K biosynthesis has been made to analyze the potential for engineering new microviridin-like or even unrelated peptides and proteins using two unprecedented ligases. MvdD, an ester ligase, has been shown to exhibit substrate tolerance to a certain degree. Bioactivity tests of the obtained mutants were not conducted [Philmus, et al., 2009]. Similarely, the nisin cyclase NisC has been shown *in vivo* to process a wide array of unrelated and designed peptides [Rink, et al., 2007].

Irrespective of the difficulties using total synthesis some attempts were made to construct potentially promising new classes of small cyclic thiazole and oxazole containing peptides, albeit with comparatively low yields. Investigations concerning their properties and application are still in progress [Haberhauer and Rominger, 2002: ; Pattenden and Thompson, 2001].

For the construction of designer peptides, leader peptide mutagenesis and manipulation of the posttranslationally modifying enzymes have been considered in literature [Field, et al., 2010]. The introduction of alternative cleavage sites at the C-terminus of the nisin leader peptide has been shown, without hampering the modifying enzymes [Plat, et al., 2010]. Designed and unrelated peptides were fused to the nisin leader and analyzed using the corresponding posttranslationally modifying enzymes, including NisBTC. Ring formation was demonstrated for most of these peptides, enabling the use of these enzymes for biotechnological production of therapeutic peptides with modulated activities and enhanced stabilities [Kluskens, et al., 2005: ; Kuipers, et al., 2004].

1.3 Microviridins

Microviridins are ribosomally synthesized cyanobacterial depsipeptides that comprise a unique and novel cage-like architecture. Microviridins are exclusively composed of 13-14 proteinogenic L-amino acids, with the one exception of the N-terminal acetic acid that is commonly present. The first microviridin, named microviridin A, has been isolated in 1990 from a bloom of *Microcystis viridis* Nies102 [Ishitsuka, et al., 1990]. Until this study started 14 microviridins were detected (Figure 1-9) and strains of *Nostoc* and *Planktothrix* were identified as additional producers [Philmus, et al., 2008: ; Reshef and Carmeli, 2006]. The highly unusual multicyclic structure is established by two ω-ester and a secondary ω-amide bond, which are formed between amino acid side chains.

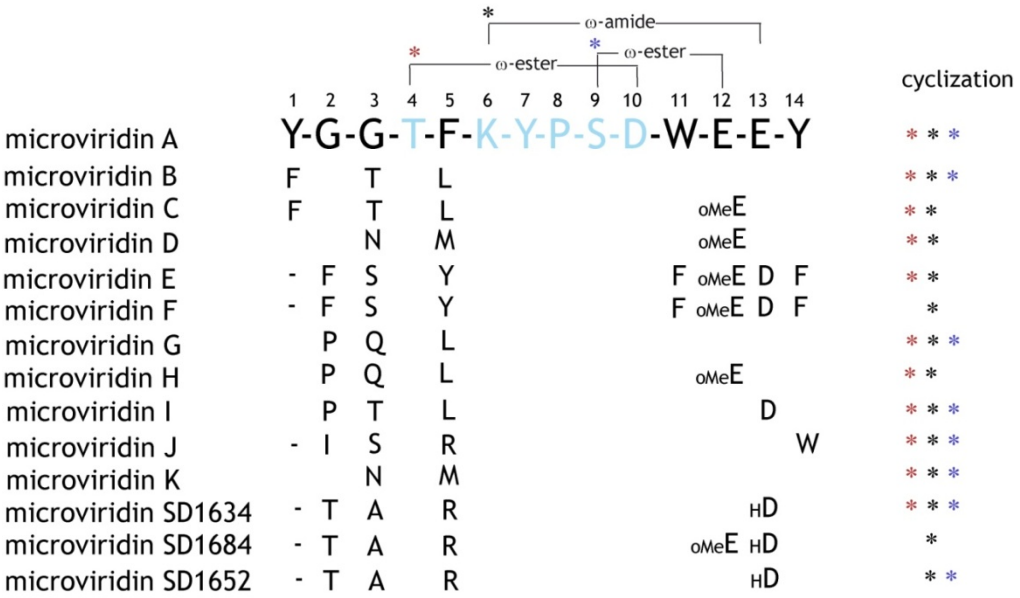

Figure 1-9: Detected microviridins with their amino acid sequence and type of cyclization

The figure shows the 14 microviridin sequences so far uncovered from cyanobacteria belonging to *Microcystis*, *Planktothrix* and *Nostoc*. The sequence of microviridin A is composed of 14 amino acids, as shown in the numbers above the sequence, and has been used to illustrate the basic structural properties. All other microviridins are shown comparatively to microviridin A, which means only differences are given. The KYPSD motif (light blue) is highly conserved among microviridins. The amino acids involved in crosslink formation are shown in black brackits. The color coded asteriks indicate the number and type of cyclization. Hyphen: not present, oMeE: methyl ester of glutamate in position 12, HD: ß-hydroxy aspartic acid.

The main peptide ring consists of seven amino acids. The large lactone ring is established between the 4-carboxy group of aspartate (D) in position 10 and the hydroxy group of threonine (T) in position 4 (Figure 1-9 and Figure 1-10). The smaller lactone ring is formed involving position 9 and 12, comprising a serine (S) and a glutamate (E) (Figure 1-9 and Figure 1-10). The lactame ring is built between the 6-amino group of lysine (K) in position 6 and the 4-carboxy group of glutamate (E) or aspartate (D) in position 13 (Figure 1-9 and Figure 1-10) [Welker and Von Döhren, 2006]. All so far detected microviridins show a highly

conserved TxKYPSD motif (with x being L, M, Y or W) in their core sequence (shown in light blue in Figure 1-9), whereas the N- and C-terminus display a higher variability. Some of the detected microviridins are bicyclic or monocyclic, whereas the majority of them are tricyclic.

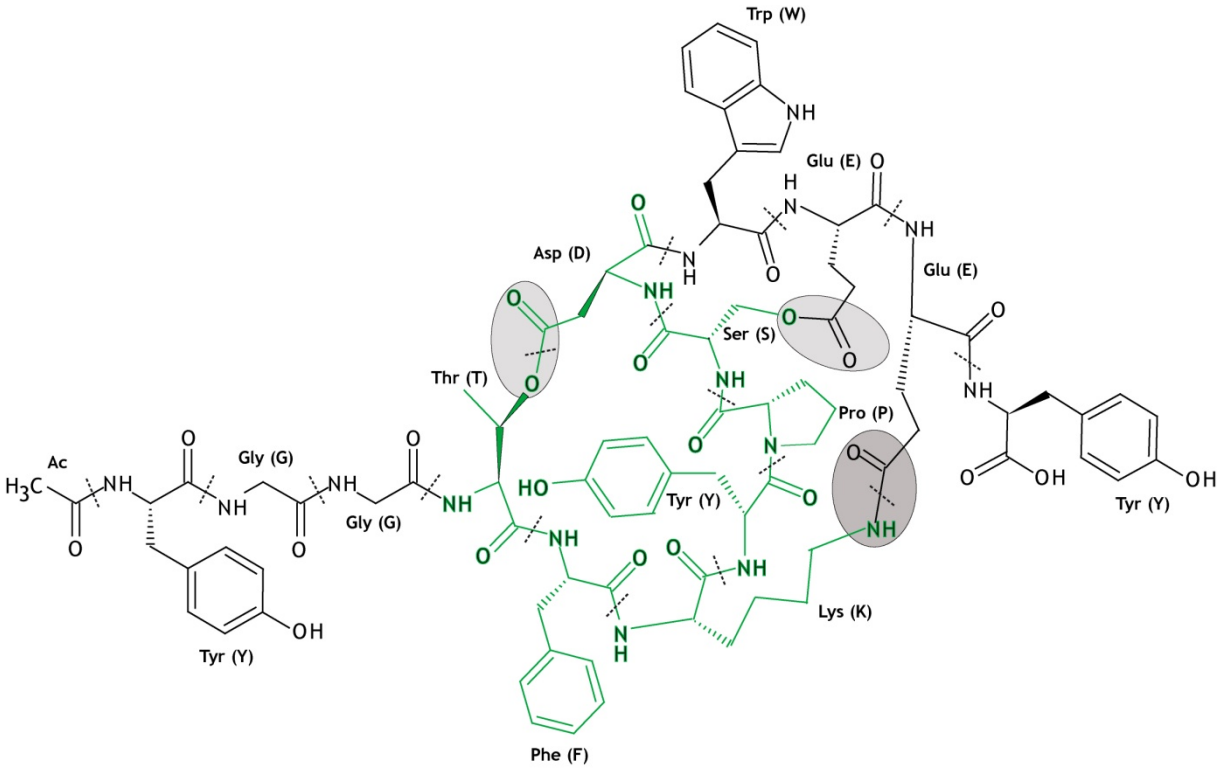

Figure 1-10: Structure of tricyclic microviridin A

The main peptide ring is shown in green. The two ester bonds are highlighted in light gray and the amide bond in darker gray. Ac: acetylation

1.3.1 The genetic basis of microviridin biosynthesis

The genetic basis of microviridin production was incipiently assumed to be a nonribosomal peptide synthetase (NRPS) as cyanopeptolins, belonging to a cyanobacterial depsipeptide family, were proven to be assembled using this biosynthetic pathway [Martin, et al., 1993: ; Tooming-Klunderud, et al., 2007]. Recently, Ziemert and coworkers showed that heterologously expressed microviridin B from *Microcystis aeruginosa* Nies298 is produced via the ribosomal peptide assembly line. A gene cluster of about 6 kb containing 5 genes, named *mdnABCDE*, was identified and supposed to be required in this constellation for the complete biosynthesis and processing of microviridins [Ziemert, et al., 2008].

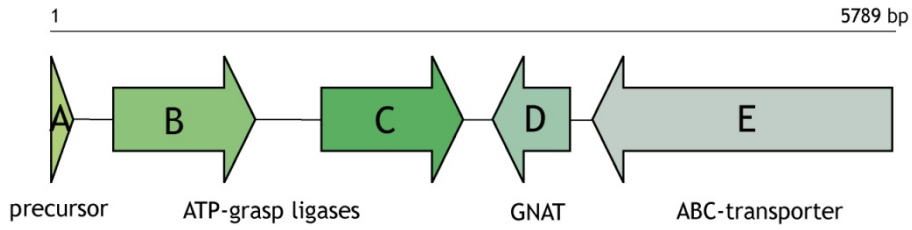

Figure 1-11: Microviridin *mdnABCDE* gene cluster for the production of microviridin B from *Microcystis aeruginosa* Nies298

The genes encode for: A) microviridin precursor, B/C) ATP-grasp ligases, D) GCN5-related N-acetyltransferase (GNAT), E) ABC (ATP-binding cassette)-transporter

1.3.2 Posttranslational modifications by two ATP-grasp ligases

MdnA encodes the precursor molecule for microviridin production. It consists of a leader peptide and a core peptide with the microviridin sequence at the C-terminus, as commonly reported for ribosomal precursor peptides (see chapter 1.2.2.1) (Figure 1-12). MdnA is processed by the tailoring enzymes encoded in the microviridin gene cluster.

The ligases MdnB and MdnC belong to a superfamily of enzymes, which is characterized through an unusual binding fold referred to as palmate or ATP-grasp fold. These enzymes conduct ATP-dependent carboxylate-amine or thiol ligase activity, whereupon primary or secondary amines might be used to create the peptide bond. Furthermore, their activity depends on the presence of Mg^{2+} and GTP or ATP. Several other enzymes, as the D-alanine-D-alanine ligases involved in cell wall biosynthesis, the glutathione synthetase responsible for glutathione biosynthesis, or the biotin carboxylase involved in fatty acid biosynthesis, are known to belong to this enzyme family. A large sequence analysis of the ATP-grasp domains showed that the amino acid residues, which interact with ATP, are highly conserved within the majority of sequences [Galperin and Koonin, 1997]. A phylogenetic study revealed the microviridin ligases create an independent branch on a phylogenetic tree and subdivide again into those belonging to the MdnC ester ligase and the MdnB amide bond forming type from different cyanobacteria and the proteobacterium *Alteromonas* [Ziemert, 2009].

MdnB and MdnC catalyze the ring closures in microviridin biosynthesis. MdnC introduces successively two lactone rings starting with the larger one. MdnB depends on the activity of MdnC and can establish the peptide bond only once the ester bond formations are completed. Microviridin production *in vivo* and *in vitro* minimally depends on the genes *mdnABC* [Philmus, et al., 2008: ; Philmus, et al., 2009: ; Ziemert, et al., 2008]. Microviridins produced by MdnABC are not processed at their N-terminus.

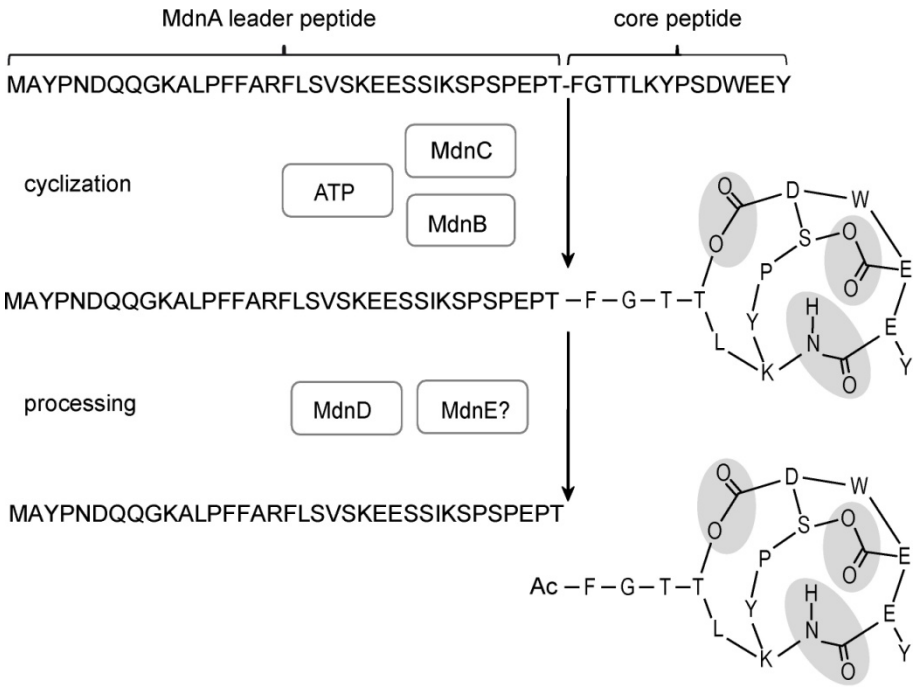

Figure 1-12: Schematic representation of microviridin B biosynthesis in *Microcystis (*similar as seen in [Ziemert, et al., 2008]

MdnC catalyzes the formation of the two lactone rings. MdnB catalyzes the formation of the lactam ring. MdnD encodes for a GNAT type acetyltransferase. MdnE encodes for an ABC-transporter. The *in vivo* function of mdnD and E was only assumed. The ester and amide bonds are highlighted in gray. Ac: acetyl group

1.3.3 An N-acetyltransferase encoded by *mdnD*

At the beginning of this study, the N-acetyltransferase MdnD, belonging to the group of the GCN5-related N-acetyltransferases (GNAT), was known to acetylate completely processed microviridins at their N-terminus *in vitro* [Philmus, et al., 2008] (Figure 1-12). *In vivo* studies have not been conducted until then. Additional functions of MdnD were not reported.

The GNAT superfamily is one of the largest enzyme families with more than 10,000 representatives today, distributed in all kingdoms. These enzymes use acyl-CoAs as a donor to acetylate their cognate substrates, which is the acceptor at the N-terminal primary amine. N-acetylation has been discussed in the past to function in a number of pathways in both prokaryotes and eukaryotes, including antibiotic resistance, transcriptional activation and DNA replication and neurohormone melatonin synthesis [Dyda, et al., 2000]. As a general benefit from prokaryotic acetylation mainly stabilization and protection of proteins against proteolytic processes has been discussed [Soppa, 2010]. An example showing the opposite function in eukaryotes has been published by Hwang and coworkers, who showed N-acetylation functions as a specific degradation signal [Hwang, et al., 2010]. Recently a bifunctional role for an N-acetyltransferase, participating in polyketide

synthesis with a decarboxylase/acetyltransferase activity has been discovered [Gu, et al., 2007]. Beside N-acetylation internal acetylation at lysine residues occurs in prokaryotes to down-regulate protein activities in phases of starvation [Yu, et al., 2008].

1.3.4 An ABC-transporter encoded by *mdnE*

MdnE, encoding for an ABC-transporter was assumed to be involved in proteolytic cleavage and/or translocation of microviridins (Figure 1-12). Microviridins were only completely processed, which means cleaved from the leader and acetylated at their N-terminus, when the entire gene cluster containing the genes *mdnABCDE* was expressed in *E. coli*. In absence of the ABC-transporter only incompletely processed microviridins were detected [Ziemert, et al., 2008].

A dual transporter peptidase function has been discussed for *Nostoc sp.* PCC7120 carrying a cryptic microviridin gene cluster and for *Alteromonas sp.* B-10-31 producing microviridin related marinostatins from a precursor molecule with a double glycine motif (see also chapter 1.2.2.3). MdnE was proposed to be the closest candidate for microviridin transport and cleavage, as it was located within the microviridin gene cluster and the removal of *mdnE* resulted in incompletely processed peptides. However, characteristic features including a recognition sequence and a peptidase domain were not detected until then [Dirix, et al., 2004: ; Michiels, et al., 2001: ; Ziemert, et al., 2008].

1.3.5 The potential of microviridins as protease inhibitors

Almost all microviridins reported so far show potent inhibitory activity *in vivo* and *in vitro* against serine proteases, such as trypsin, chymotrypsin and elastase. Some microviridins are active already in the nanomolar range (Table 1-1). Additionally microviridin J has been shown to exhibit toxicity against daphnia by causing a lethal disruption in the molting process of this zooplankton [Rohrlack, et al., 2003].

Proteases are involved in many biological processes, including food digestion, signaling cascades and lysosome associated degradation. They catalyze the irreversible disruption of peptide bonds and therefore necessitate a strict regulation and control by their corresponding naturally occurring inhibitors. Protease inhibitors are classified into serine (serpin), cysteine, aspartyl, threonine and metalloprotease inhibitors with some of them interfering with different types of proteases. As malfunction in protease production and regulation in human systems cause severe effects on the physical health, there is a major interest on the identification of potent protease inhibitors useful as pharmaceutical drugs. Several inhibitors, including those for the treatment of HIV and those to reduce blood

pressure are already in use, whereas other highly potent candidates are in clinical trials. Therapeutic agents against hepatitis C, cancer and diabetis mellitus are currently in phases I-III [Fear, et al., 2007]. Protease inhibitors are further discussed as future antibiotics. As almost all antibiotics in use target the bacterial cell-wall or protein biosynthesis, a different mode of action to overcome the emergence of antibiotic resistance guides drug generation to bacterial proteinase inhibitors [Travis and Potempa, 2000].

Not all of the protease inhibitors currently in pharmaceutical use are nonhazardous. Alpha-1-antitrypsin is a human glycoprotein. It is isolated from pooled human plasma of assured healthy donors. This proteinase inhibitor is indicated for chronic replacement therapy of patients having a congenital alpha-1-antitrypsin deficiency, leading to progressive pulmonary emphysema. There is no procedure available for a guaranteed removal of all viral infectivity from pooled human plasma (http://www.talecris-pi.info/inserts/Prolastin-C.pdf). The scarcity of the raw material, the challenging manufacturing procedure [Reh, et al., 2002], the insufficient safety towards unknown pathogens and the need for patients to administer an weekly intravenous dosage demands new and better elastase inhibitors. Multiple efforts to generate recombinant human alpha-1-proteinase inhibitors have been reported. Heterologous production resulted in aberrant glycosylation patterns, which is considered to decrease protein stability with the result of rapid clearance from the circulation. So far not a single recombinant alpha-1-antitrypsin acquired therapeutic importance [Karnaukhova, et al., 2006].

Microviridins, produced from a bacterial origin, have high therapeutic potential as alternative candidate drugs for elastase inhibition [Murakami, et al., 1997: ; Okino, et al., 1995: ; Ziemert, et al., 2008]. Chymotrypsin inhibitory activity is discussed in literature for an application in the elucidation of pancreatitis pathogenesis [Imada, et al., 1986].

The following table represents an overview of naturally occuring microviridin bioactivities.

Table 1-1: Selected data about the inhibitory activities of known microviridins (similar as seen in [Ziemert, et al., 2010])

		inhibitory activity (IC_{50}) in µmol/l			
microviridin	mass (Da)	elastase	chymotrypsin	trypsin	reference
A	1.730	>58	>58	>58	[Ishitsuka, et al., 1990]
B	1.722	0.026	1.5	34	[Okino, et al., 1995]
C	1.754	0.048	2.8	18	[Okino, et al., 1995]
D	1.802	0.388	0.7	>55	[Shin, et al., 1996]
E	1.666	0.360	0.7	>60	[Shin, et al., 1996]
F	1.682	3.4	>59	>59	[Shin, et al., 1996]
G	1.805	0.010	0.78	>55	[Murakami, et al., 1997]
H	1.837	0.017	1.6	>54	[Murakami, et al., 1997]
I	1.764	0.193	nd	nd	[Fujii, et al., 2000]
J	1.684	>6.0	1.7	0.020-0.090	[Rohrlack, et al., 2003]
SD1684	1.684	nd	inactive	inactive	[Reshef and Carmeli, 2006]
SD1634	1.634	nd	15.7	8.2	[Reshef and Carmeli, 2006]
SD1652	1.652	nd	inactive	inactive	[Reshef and Carmeli, 2006]

nd: not determined; >: no inhibitory activity has been detected below this concentration

1.3.6 Physicial mechanism and ecological function of microviridins

The mode of action of microviridins is still a secret. There are no details or systematic studies about the precise physicial mechanism or preferential target specificities for these protease inhibitors. It might be due to the fact that microviridins and generally the ribosomally synthesized peptides just came into the center of interest several years ago. Commonly, it is known that proteolytic inhibitors use two strategies including either covalent trapping or a reversible tight-binding reaction to the active site of the target [Rawlings, et al., 2004].

The main focus following the isolation of a novel compound is concentrated on the bioactivity in terms of promising commercial application as a drug to cure or at least relieve human diseases. Very little is known about the fundamental role of microviridin in the physiology and ecology of the producing cyanobacterium. Microviridin J shows toxicity to daphnia by lethally disrupting the molting process, which has an impact on the whole ecosystem. Based on that information, microviridin has been proposed as a compound of chemical defense against these grazers [Rohrlack, et al., 2004]. In a study conducted by [Makower, 2010], microviridins have been assumed to be rather involved into intracellular processes then acting in quorum sensing based interspecies interactions.

For the majority of cyanobacterial compounds the function and benefit for the producer remains unknown. A few studies considered these metabolites as allelochemicals influencing the growth and development of neighboring species, including green algaes [Mason, et al., 1982], plants [Hirata, et al., 2003] and cyanobacteria [Jüttner, et al., 2001: ; Singh, et al., 2001].

2 Thesis objectives

The main objective for this study was to analyze a new microviridin gene cluster from *Microcystis aeruginosa* Nies843, to characterize and manipulate the biosynthetic pathway of these unique ribosomally synthesized tricyclic compounds. In general, bioinformatic analysis of microviridin gene clusters suggested a novel type of processing machinery encoded in the microviridin genes *mdnABCDE* compared to other ribosomally synthesized peptides. Two ATP-grasp ligases, MdnB and MdnC, were previously shown to sequentially introduce ω-ester and ω-amide bonds into the linear precursor peptide MdnA to yield the rare and fascinating cage-like architecture [Philmus, et al., 2008: ; Philmus, et al., 2009].

This work was aimed on the construction of a minimal expression system to gain new insight into the mechanism of microviridin production. One of the goals was to investigate the leader peptide of MdnA by bioinformatic and mutational strategies for the presence of recognition motifs for the ATP-grasp ligases, the ABC-transporter and/or another peptidase. The proposed function of MdnD as an N-acetyltransferase ought to be confirmed *in vivo* by knockout mutagenesis. About the role of MdnE has been speculated as a transporter peptidase before [Ziemert, et al., 2008]. To clarify this assumption, the subcellular localization of microviridins and the cleavage from the leader peptide had to be analyzed in presence and absence of MdnE and by mutagenesis in the Walker A motif of the ATP-binding domain. An *in vitro* reconstitution of microviridin biosynthesis and initial studies of protein interactions were intended.

Another goal was to analyze the microviridin core motif in terms of flexibility for amino acid exchanges that could guide the creation of compound libraries with improved and redesigned bioactivities. Bioactivity tests for the constructed mutants were intended. Furthermore, it was destined to exchange the Nies843 precursor peptide against examples taken from the natural library.

3 Experimental design

3.1 Initial studies into the mechanism of microviridin production

Initial studies of the mechanism behind microviridin production were made using a fosmid expression system and Duet vectors, alone or in combination of both systems, as given in the following schematic.

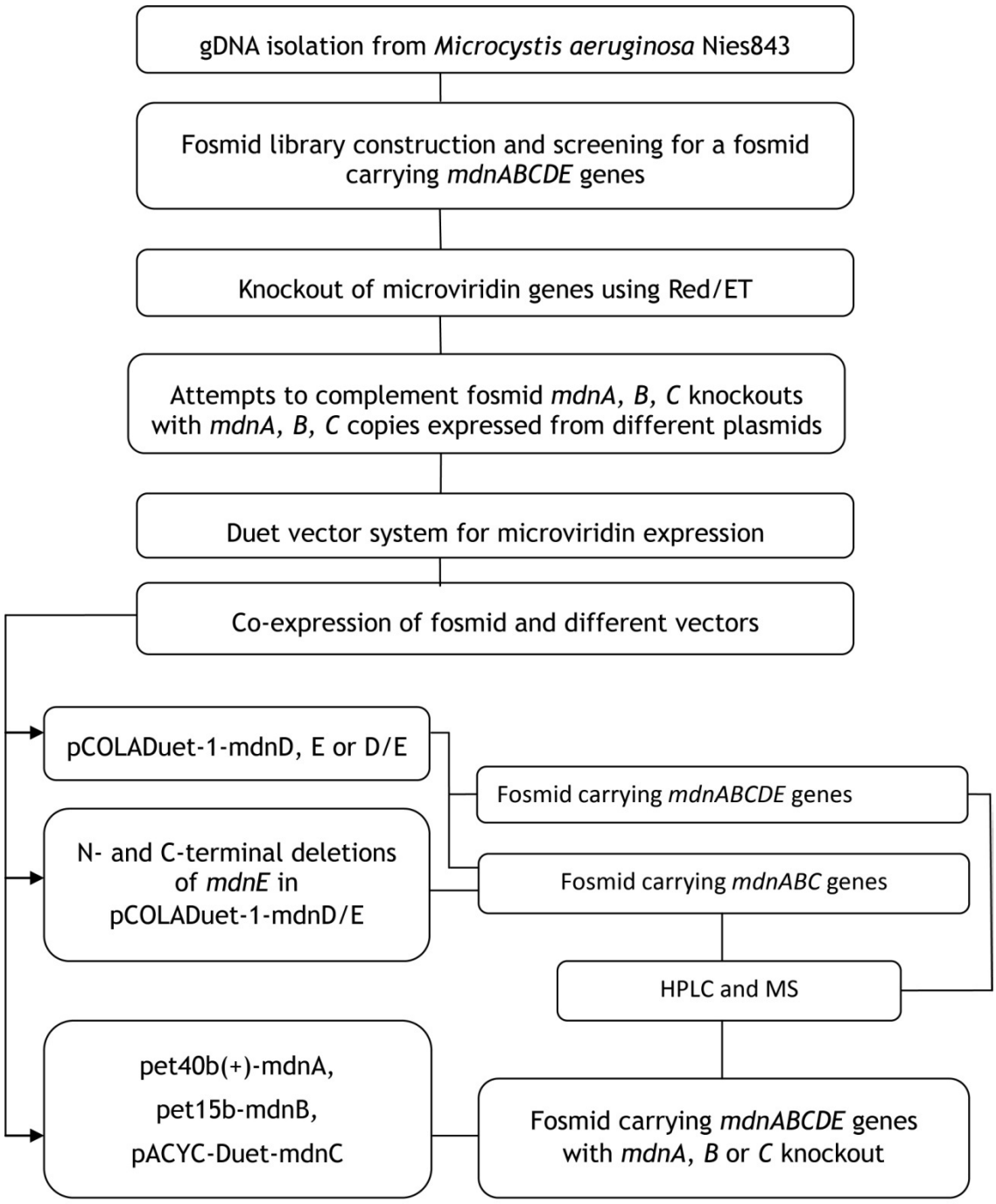

Figure 3-1: Fosmids and Duet vectors to analyze microviridin production

3.2 Advanced studies into the mechanism of microviridin production

A small microviridin expression platform was constructed and served as a template for a site-directed mutagenesis approach in the genes *mdnA, B* and *E*. Protein interactions were studied using SPR. The role of the ABC-transporter in microviridin L biosynthesis was determined to estimate his function in transport and as a putative scaffolding protein, as presented in the following diagram.

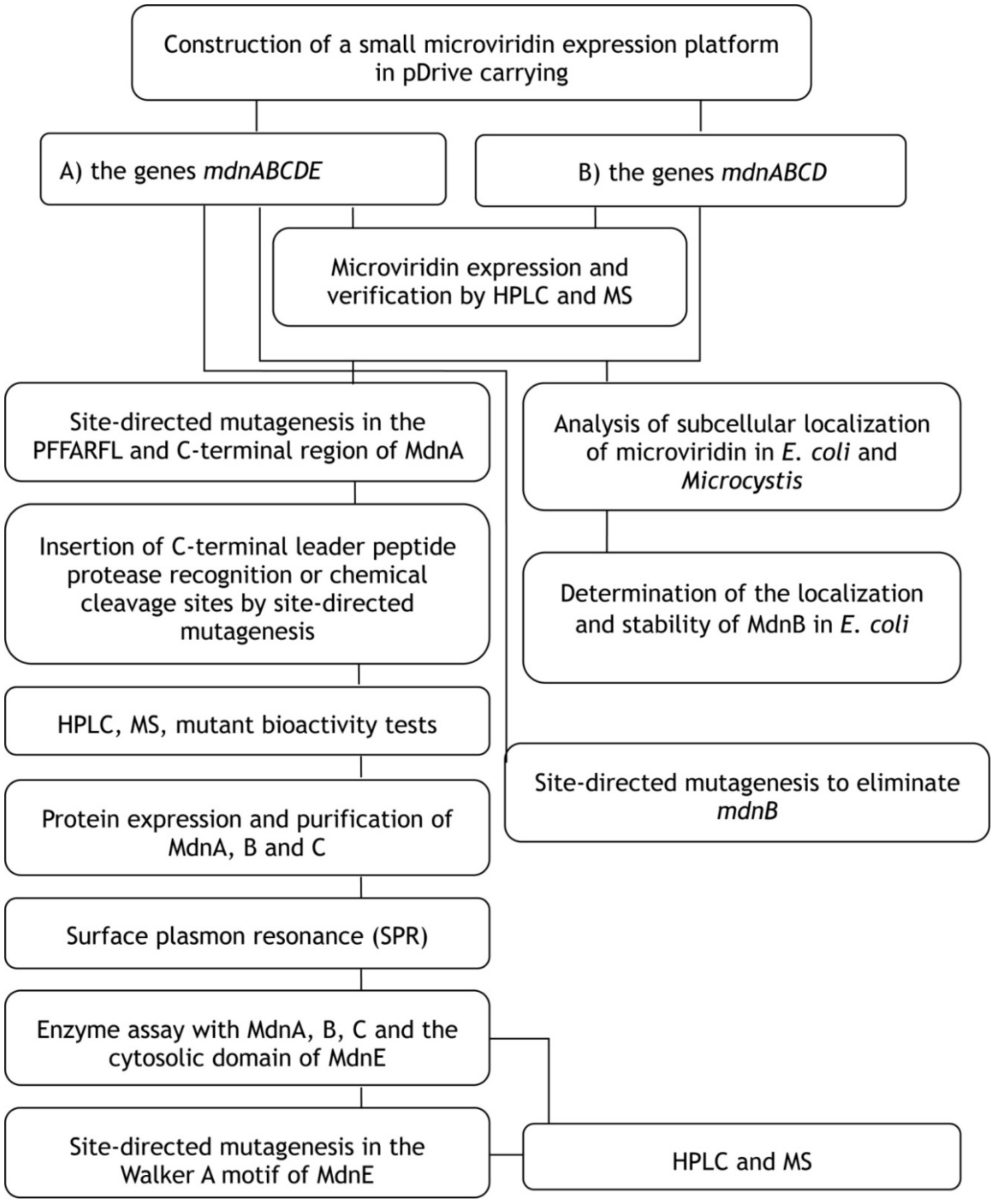

Figure 3-2: Advanced studies into the mechanism of microviridin biosynthesis

3.3 Compound driven analysis of microviridin production

The microviridin precursor molecule has been manipulated in the highly conserved part of the coding region in order to determine the flexibility against amino acid exchanges and the influence on the bioactivity of the resulting compounds. A complete exchange of the precursor molecule as well as certain amino acid exchanges should show the potential to create microviridin peptide libraries. Figure 3-3 presents an overview of the procedure.

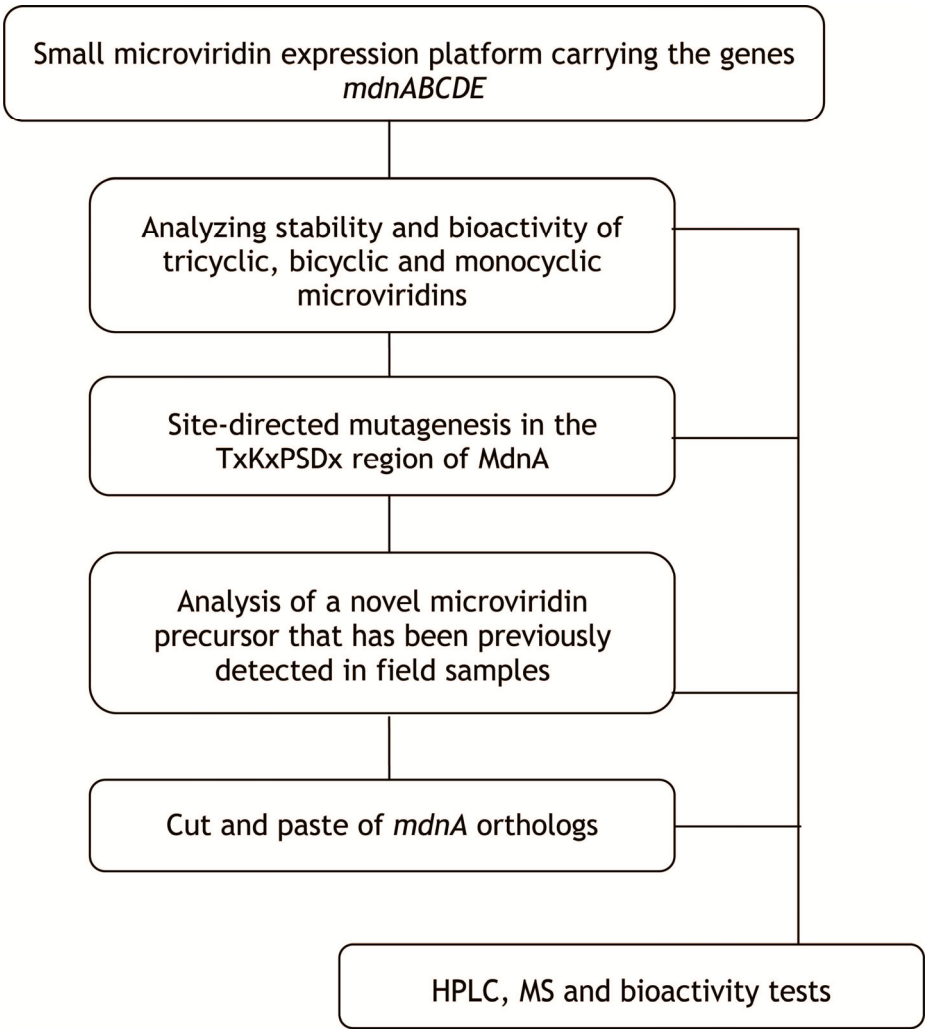

Figure 3-3: Manipulation of the microviridin coding region and expression of cryptic precursor molecules

4 Materials

4.1 Bacterial strains

All bacterial strains used in this study are listed in the following table.

Table 4-1: Bacterial strains

bacterial strains	description/origin	reference
Microcystis aeruginosa		
Nies843	toxic, bloom-forming, microviridin L producer/ Lake Kasumigaura Ibaraki Japan, 1997	[Kaneko, et al., 2007], [Ziemert, et al., 2010]
Nies298	toxic, bloom-forming, microviridin B producer/ Lake Kasumigaura, Ibaraki, Japan, 1982	[Cadel-Six, et al., 2008]
PCC7806	toxic, bloom-forming/ Braakman reservoir, The Netherlands, 1972	Pasteur Culture Collection, Paris, France
Escherichia coli		
BL21 (DE3)	chemically or electro competent cells for heterologous expression	Merck Chemicals Ltd., Nottingham, UK
Top10	chemically competent cells for routine cloning procedures	Invitrogen, Carlsbad, CA, USA
XL1 Blue	chemically competent cells for routine cloning procedures	Agilent Technologies, La Jolla, CA, USA
XL10 Gold	ultra competent cells in use with Quik change II XL Site-Directed Mutagenesis Kit	Agilent Technologies, La Jolla, CA, USA
Epi300T1R	fosmid library host strain	Epicentre Biotechnologies, Madison, WI, USA

4.2 Genomic DNA

Genomic DNA of *Cyanothece sp.* PCC7822 and *Nostoc sp.* PCC7120 (Pasteur Culture Collection, Paris, France) has been used for PCR amplification of *mdnA* orthologs.

4.3 Plasmids and fosmids

pACYCDuet-1 Merck Chemicals Ltd., Nottingham, UK

pETDuet-1 Merck Chemicals Ltd., Nottingham, UK

pCOLADuet-1	Merck Chemicals Ltd., Nottingham, UK
pET40b(+)	Merck Chemicals Ltd., Nottingham, UK
pET15b	Merck Chemicals Ltd., Nottingham, UK
pCC1FOS	EPICENTRE Biotechnologies, WI, USA
pDrive	Qiagen, Hilden, Germany
pRedET	Gene Bridges GmbH, Heidelberg, Germany
MRC-derived fosmid	[Ziemert, et al., 2008]

4.4 Media

Bacto agar	BD, Sparks, MD, USA
Bg11	[Rippka, et al., 1979]
LB-agar	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
LB-broth	CARL ROTH GMBH + CO. KG, Karlsruhe, Germany
LB-broth +10mM $MgSO_4$	25 g/ L LB-broth, 10 mM $MgSO_4$, autoclaved
M9 medium with thiamine-HCl and L-leucine	200 ml M9 salt solution (64 gNa_2HPO_4 x 2 H_2O, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl per liter), 2 ml 1 M $MgSO_4$, 20 ml 20 % (w/ v) glucose, 100 µl $CaCl_2$, 1 g thiamine-HCl, 60 mg L-leucine, adjusted to 1 liter, separately autoclaved or filter sterilized
E. coli minimal medium	A) salt solution: 13.3 g/l KH_2PO_4, 4.0 g/l $(NH_4)_2HPO_4$, 1.7 g/l citric acid, 60 mg/l Fe-III-citrate, 2.5 mg/l $CoCl_2x6H_2O$, 15.0 mg/l $MnCl_2x4H_2O$, 1.5 mg/l $CuCl_2x2H_2O$, 2.5 mg/l $Na_2MoO_4x2H_2O$, 8.0 mg/l $Zn(CH_3COO)_2x2H_2O$, 3.0 mg/l H_3BO_3, 8.4 mg/l EDTA; B) 1.2 g/l $MgSO_4x7H_2O$; C) 25 g/l glucose; D) 4.0 mg/l thiamine-HCl; E) 50 mg/l L-leucine; pH 6.7-7.0
NZY^+-broth	10 g N-Z-amine A, 5 g yeast extract, 5 g NaCl, pH 7.5, adjusted to 1 liter; additionally added: 12.5 ml 1 M $MgCl_2$, 12.5 ml 1 M $MgSO_4$, 10 ml of 2 M glucose

4.5 Antibiotics

Ampicillin-sodium salt	CARL ROTH, Karlsruhe, Germany
Chloramphenicol	CARL ROTH, Karlsruhe, Germany
Kanamycin sulfate	CARL ROTH, Karlsruhe, Germany
Streptomycin sulfate	CARL ROTH, Karlsruhe, Germany
Tetracycline-hydrochloride	CARL ROTH, Karlsruhe, Germany

4.6 Primers

DNA oligonucleotides were purchased from Sigma-Genosys (Steinheim, Germany) or Eurofins MWG Operon (Ebersberg, Germany). A summary of primers used in this study is given in the appendix.

4.7 Markers and dyes

6 x DNA Loading Dye	Fermentas Life Sciences, St. Leon-Rot, Germany
Lambda DNA-*Pst*I digest	Fermentas Life Sciences, St. Leon-Rot, Germany
PageRuler Prestained Protein Ladder	Fermentas Life Sciences, St. Leon-Rot, Germany
5x SDS-loading dye with 2-mercaptoethanol	250 mM Tris pH 6.8, 0.5 % bromphenol blue, 10 % (w/v) SDS, 50 % (w/v) glycerol, 500 mM 2-mercaptoethanol

4.8 Enzymes

Factor Xa Protease	Promega Corporation, Madison, WI, USA
Lysozyme from chicken egg white	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Pfu DNA polymerase	Qiagen, Hilden, Germany
*Pfu*Ultra HF DNA polymerase	Agilent Technologies, Waldbronn, Germany
Phusion High-Fidelity DNA polymerase	Thermo Fisher Scientific GmbH, Dreieich, Germany
Proteinase K	Fermentas Life Sciences, St. Leon-Rot, Germany
restriction endonucleases	Fermentas Life Sciences, St. Leon-Rot, Germany; New England Biolabs, Frankfurt/Main, Germany
RNase A/T1 Mix	Fermentas Life Sciences, St. Leon-Rot, Germany
Sequencing grade modified trypsin	Promega GmbH, Mannheim, Germany

T4 DNA ligase	Fermentas Life Sciences, St. Leon-Rot, Germany
Taq DNA polymerase	Qiagen, Hilden, Germany
Thermus aquaticus DNA polymerase	Kindly provided by the lab of Prof. C. Schmitz-Linneweber, Humboldt University of Berlin, Germany; [Desai and Pfaffle, 1995]

4.9 Chemically synthesized peptides

The microviridin precursor sequences including MvdE from *Planktothrix agardhii* (MSKNVKVSAPKAVPFFARFLAEQAVEANNSNSAPYGNTMKYPSDWEEY), MdnA(1)-original Nies843 precursor (MSKNVKVSAPKAVPFFARFLAEQAVEANNSNSAPYGNTMKYPSDWEEY), MdnA(2)—triple PFFARFL manipulated precursor from Nies843 (MAYPNDQQGKALPGFLRALSVSKEESSIKSPSPEPTYGGTFKYPSDWEDY) and MdnA-original Nies298 precursor (MAYPNDQQGKALPFFARFLSVSKEESSIKSPSPEPTFGTTLKYPSDWEEY) from *Microcystis aeruginosa* strains Nies298 and Nies843 were purchased from Genescript, NJ, USA.

4.10 Proteins

Mvn	GenBank: CAO86269.1; kindly provided by Dr. Jan-Christoph Kehr, group of Prof. Dittmann, University of Potsdam, Germany
ActM	GenBank: CAM57974.1, kindly provided by Arthur Guljamow, group of Prof. Dittmann, University of Potsdam, Germany
TusA	[Ikeuchi, et al., 2006]; kindly provided by Angelika Lehmann, group of Prof. Leimkühler, University of Potsdam, Germany
POD	Sigma-Aldrich Chemie Gmbh, Munich, Germany
BSA (Albumin Fraktion V)	CARL ROTH, Karlsruhe, Germany

4.11 Chemicals

2-Mercaptoethanol	Ferak-Berlin GmbH, Berlin, Germany
Acetic acid	CARL ROTH, Karlsruhe, Germany
Acetonitrile ROTISOLV HPLC grade	CARL ROTH, Karlsruhe, Germany
Ammonium hygrogen carbonate (NH_4HCO_3)	Sigma-Aldrich Chemie Gmbh, Munich,

	Germany
APS	CARL ROTH, Karlsruhe, Germany
ATP	Fermentas Life Sciences, St. Leon-Rot, Germany
Biozym-LE-Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Boric acid	CARL ROTH, Karlsruhe, Germany
Chloroform	CARL ROTH, Karlsruhe, Germany
Citric acid	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Cobald(II) chloride	ICN Biomedicals Inc., Ohio, USA
Copper chloride	CARL ROTH, Karlsruhe, Germany
CopyControl Induction Solution	EPICENTRE Biotechnologies, Madison, WI, USA
D (+)-sucrose	CARL ROTH, Karlsruhe, Germany
D-Glucose	Merck, Darmstadt, Germany
Diammonium hydrogen phosphate $(NH_4)_2HPO_4$	CARL ROTH, Karlsruhe, Germany
DMSO	CARL ROTH, Karlsruhe, Germany
Di-potassium hydrogen phosphate trihydrate $(K_2HPO_4 \times 3\ H_2O)$	CARL ROTH, Karlsruhe, Germany
Di-sodium hydrogen phosphate $(Na_2HPO_4 \times 2\ H_2O)$	CARL ROTH, Karlsruhe, Germany
D-Maltose	SERVA Electrophoresis GmbH, Heidelberg, Germany
dNTP Mix, 10 mM each	Fermentas Life Sciences, St. Leon-Rot, Germany
Ethanol	Berkel AHK Alkoholhandel, Berlin, Germany
Ethidium bromide	Sigma-Aldrich Chemie Gmbh, Munich, Germany
EDTA	CARL ROTH, Karlsruhe, Germany
Gel-Code Blue Safe Protein stain	Thermo Fisher Scientific GmbH, Dreieich, Germany
Glycerol	CARL ROTH, Karlsruhe, Germany
HEPES	CARL ROTH, Karlsruhe, Germany
Hydrochloric acid 37 %	CARL ROTH, Karlsruhe, Germany
Hydroxylamine hydrochloride	FLUKA Feinchemikalien GmbH, Neu-Ulm, Germany

Imidazol	CARL ROTH, Karlsruhe, Germany
IPTG	CARL ROTH, Karlsruhe, Germany
Isoamylalcohol	CARL ROTH, Karlsruhe, Germany
Isopropanol	CARL ROTH, Karlsruhe, Germany
L-arabinose	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Liquid nitrogen	AIR LIQUIDE Deutschland GmbH, Berlin, Germany
Magnesium sulfate	CARL ROTH, Karlsruhe, Germany
Magnesium chloride	CARL ROTH, Karlsruhe, Germany
Methanol ROTISOLV HPLC gradient grade	CARL ROTH, Karlsruhe, Germany
Monosodium phosphate-2-hydrate ($NaH_2PO_4x2H_2O$)	CARL ROTH, Karlsruhe, Germany
N,N-Dimethylformamide (DMF)	Merck, Darmstadt, Germany
Ni-NTA agarose	Qiagen, Hilden, Germany
Nonidet P-40	Sigma-Aldrich Chemie Gmbh, Munich, Germany
N-Z-amine A	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Phenol-Chloroform-Isoamylalcohol 25:24:1	CARL ROTH, Karlsruhe, Germany
PMSF	CARL ROTH, Karlsruhe, Germany
PIPES	CARL ROTH, Karlsruhe, Germany
Potassium chloride	SERVA Electrophoresis GmbH, Heidelberg, Germany
potassium dihydrogen phosphate (KH_2PO_4)	CARL ROTH, Karlsruhe, Germany
Protein Assay	Bio-Rad, Munich, Germany
Rotiphorese Gel30 (37.5:1)	CARL ROTH, Karlsruhe, Germany
SDS ultra pure	CARL ROTH, Karlsruhe, Germany
Skim milk powder	FLUKA Feinchemikalien GmbH, Neu-Ulm, Germany
Sodium acetate	CARL ROTH, Karlsruhe, Germany
Sodium azide	CARL ROTH, Karlsruhe, Germany
Sodium carbonate anhydrous	CARL ROTH, Karlsruhe, Germany
Sodium chloride	CARL ROTH, Karlsruhe, Germany
Sodium hydroxide	CARL ROTH, Karlsruhe, Germany
Sodium molybdate	CARL ROTH, Karlsruhe, Germany
sorbitol	CARL ROTH, Karlsruhe, Germany
S-protein agarose	Merck Chemicals Ltd., Nottingham, UK

TEMED	CARL ROTH, Karlsruhe, Germany
TFA	FLUKA Feinchemikalien GmbH, Neu-Ulm, Germany
Thiamine-HCl	SERVA Electrophoresis GmbH, Heidelberg, Germany
Tris	CARL ROTH, Karlsruhe, Germany
Triton X	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Tween20	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Urea	M.P. Biomedicals Inc., Eschwede, Germany
X-Gal	CARL ROTH, Karlsruhe, Germany
Yeast extract	SERVA Electrophoresis GmbH, Heidelberg, Germany
Zink acetate	SERVA Electrophoresis GmbH, Heidelberg, Germany

4.12 Buffer compositions

All buffer components were calculated for the volume of 1 liter. Sometimes stock solutions were prepared and later diluted to the concentration needed in the appropriate experiment. The pH was adapted using HCl 37 %, NaOH or acedic acid.

10x PBS pH 7.4	80 g NaCl, 2.0 g KCl, 17.8 g Na_2HPO_4 x 2 H_2O, 2.4 g KH_2PO_4, H_2O
10x PBS-T pH 7.4	80 g NaCl, 2.0 g KCl, 17.8 g Na_2HPO_4 x 2 H_2O, 2.4 g KH_2PO_4, 10 ml Tween20, H_2O
10x SDS running buffer	30.3 g Tris base, 144 glycine, 10 g SDS, H_2O
10x TBS-T	500 ml 1 M Tris-HCl pH 7.5, 300 ml 5 M NaCl, 10 mL Tween 20, 190 ml H_2O
1x Transfer buffer with methanol	200 ml 5 x transfer buffer, 200 ml methanol, 600 ml H_2O
50x TAE pH 8.0	242 g Tris base, 57.1 g acetic acid, 100 ml EDTA pH 8.0, H_2O
5x Transfer buffer	15 g Tris base, 72 g glycine, H_2O
Buffer B	100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, H_2O, pH 8.0

$CaCl_2$-solution	60 mM $CaCl_2$, 15 % glycerol, 10 mM PIPES, H_2O, pH 7.0
Elution buffer pH 8.0	50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazol, H_2O
Factor Xa reaction buffer	20 mM Tris-HCl, 50 mM NaCl, 1 mM $CaCl_2$, H_20, pH 6.5
HEPES 0.1 M, KCl, NaCl, pH 8.0	23.8 g HEPES, 0.2 g KCl, 8.0 g NaCl, H_2O
Lysis buffer pH 8.0	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, H_2O
P1	50 mM Tris-HCl,10 mM EDTA, H_2O, pH 8.0
P2 (lysis buffer)	200 mM NaOH, 1 % SDS, H_2O, pH 8.0
P3 (neutralization buffer)	3 M sodium acetate, H_2O, pH 5.5
Phage-dilution buffer	10 ml 1 M Tris-HCl pH 8.3, 5.84 g NaCl, 2.0 g $MgCl_2$ x 6 H2O, H_2O
Plasmolysis buffer for cyanobacteria	91.1 g sorbitol, 10 ml 1 M Tris-HCl pH 7.6, 116 mg NaCl, H_2O
Separating gel buffer pH 8.8	750 mM Tris pH 8.8, 0.2 % SDS, H_2O
Sodium acetate buffer 10 mM, pH 4.0	0.82 g sodium acetate, H_2O
Stacking gel buffer pH 6.8	250 mM Tris pH 6.8, 0.2 % SDS, H_2O
TE-buffer pH 8.0	10 ml 1 M Tris-HCl pH 8.0, 2 ml 0.5 M EDTA pH 8.0, H_2O
TES buffer	50 mM Tris-HCl, 100 mM EDTA, 25 % sucrose, H_2O, pH 8.0
Tris-HCl 1 M pH 6.8-8.0	121.14 g Tris, H_2O
Tris-HCl-30 % sucrose pH 8.0	30 ml Tris-HCl 1 M pH 8.0, 300 g D (+)-sucrose, H_2O
Wash buffer pH 8.0	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazol, H_2O

4.13 Kits

CopyControl Fosmid Library Production Kit	EPICENTRE Biotechnologies, Madison, WI, USA
Counter-Selection BAC Modification Kit	Gene Bridges GmbH, Heidelberg, Germany
GeneJET PCR Purification Kit	Fermentas Life Sciences, St. Leon-Rot, Germany
JETSORB Gel Extraction Kit	Genomed, Löhne, Germany
QIAGEN PCR Cloning Kit	Qiagen, Hilden, Germany

QIAGEN Plasmid Mini Kit	Qiagen, Hilden, Germany
Quik Change II XL Site-Directed Mutagenesis Kit	Agilent Technologies, Waldbronn, Germany
ΛE3 Lysogenization Kit	Merck, Darmstadt, Germany

4.14 Membranes, filters, cartridges, chip

Acrodisc 4mm Syringe Filter, 0.45 µm Nylon membrane	Pall, Dreieich, Germany
Amersham Hybond-C Extra	GE Healthcare Europe GmbH, Munich, Germany
Amicon Ultra-0.5 ml 30K	Millipore GmbH, Schwalbach/Ts., Germany,
CM5 sensor chip	GE Healthcare Europe GmbH, Munich, Germany
Glas beads, 0.1-0.11 mm	Sartorius AG, Göttingen, Germany
Glas beads, 0.25-0.50 mm	CARL ROTH, Karlsruhe, Germany
Kimwipe tissue	KIMBERLY-CLARK PROFESSIONAL, Rosewell, GA, USA
Polypropylene columns (1 ml)	Qiagen, Hilden, Germany
Sep-Pak Plus C18 cartridge 55-105 µm	Waters GmbH, Eschborn, Germany
Syringe filters FP30/0.2 CA-S, 0.2 µm	Whatman GmbH, Maidstone, UK
Syringe filters FP30/0.45 CA-S, 0.45 µm	Whatman GmbH, Maidstone, UK
Visking dialysis tubing 20/32, diameter: 16 mm	SERVA Electrophoresis GmbH, Heidelberg, Germany
Whatman chromatography paper 3 mm	Whatman GmbH, Maidstone, UK

4.15 Antibodies

Anti-MdnB produced in rabbit, polyclonal	[Ziemert, 2009]
Anti-rabbit IgG horseradish peroxidase conjugate, produced in goat	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Monoclonal Anti-poly histidine antibody produced in mouse	Sigma-Aldrich Chemie Gmbh, Munich, Germany
Mouse IgG, horseradish peroxidase linked whole antibody (from sheep)	GE Healthcare Europe GmbH, Munich, Germany
s-protein HRP conjugate	Merck Chemicals Ltd., Nottingham, UK

4.16 Installations and special equipment

BIACORE 2000	GE Healthcare Europe GmbH, Munich,

	Germany
TE 22 Mini Transfer Tank	Hoefer, Inc., Holliston, MA, USA
Gel Doc XR with Quantity One 4.6.2 software	Bio-Rad, Munich, Germany
Gene Pulser	Bio-Rad, Munich, Germany
Lumi-Imager with LumiAnalyst software	Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim am Rhein,Germany
Mini-Protean system	Bio-Rad, Munich, Germany
Nanodrop ND-1000 with ND V 3.7.0 software	PEQLAB Biotechnologie GMBH, Erlangen, Germany
PU 8625 UV/VIS	Philips, Hamburg, Germany
RP-HPLC consisting of SCL-10A system controller LC 10AD liquid chromatograph FCV-10AL, DGU-14A degasser SPD-M10A diode array detector SIL 10AD auto injector FRC 10A fraction collector Columns: SymmetrieShield Sentry Guart column (3.5 µm, 3.9 x 20 mm) and a SymmetrieShield RP18 (3.5 µm, 4.6 x 100 mm) Solvent A: water-TFA 0.05 % Solvent B: acetonitrile-TFA 0.05 % Software: Class-VP	Shimadzu Europa GmbH, Duisburg, Germany
Savant SC210A Speedvac concentrator with MZ2C pump	Thermo Fisher Scientific GmbH, Dreieich, Germany; Germany; VACUUBRAND GMBH + CO KG, Wertheim, Germany
T3000 Thermocycler	Biometra GmbH, Göttingen, Germany
Ultrasonic homogenizer UW60	Brandelin electronic, Berlin, Germany

5 Methods

5.1 Media and cultivation of cyanobacteria

Cyanobacteria were cultured under permanent low light conditions of about 30 µmol photons $m^{-2}s^{-1}$ at 23 °C in BG11 medium [Rippka, et al., 1979]. Cultures were grown under gentle agitation of 110 rpm.

5.2 Media and cultivation of *E. coli* cultures

E. coli cultures were grown under standard conditions at 30 °C or 37 °C and 220 -240 rpm in LB-medium for at least 5 hours to overnight. Solid agar plates contained 1.5 % agar and were grown overnight under similar conditions but without agitation.

E. coli Top10 and XL1 Blue cells were used for routine cloning procedures and for the expression of any microviridin expression platform vector. *E. coli* BL21 (DE3) cells were used for heterologous expression of proteins from Duet-1, pET15b or pET40b(+) vectors. Epi300T1R was used as a host strain for the fosmid library and for co-expression experiments with fosmids and plasmids. *E. coli* XL10 Gold cells were used in combination with the Quik change XLII mutagenesis kit following the manufacturing company's recommendations.

LB medium was used for the recovery of *E. coli* following transformation.

LB was supplemented with the appropriate amount of antibiotics to maintain a plasmid and to select for resistance, as shown in the following table.

Table 5-1: Antibiotic concentrations

plasmid/fosmid	antibiotic	mg/l liquid and solid LB
pCC1FOS	chloramphenicol	12.5
pCC1FOS knockouts	chloramphenicol	12.5
	kanamycin	50
	streptomycin	15
pACYCDuet-1	chloramphenicol	34
pCOLADuet-1	kanamycin	30
pETDuet-1	ampicillin	50
pET40b(+)	kanamycin	30
pET15b	ampicillin	50
pRedET	tetracycline	3

5.3 Glycerol stocks

Frozen stocks of all constructs and strains, except cyanobacterial strains, were prepared in LB containing 50 % glycerol. Tubes were stored at -80 °C.

5.4 Total genomic DNA preparation from cyanobacteria

25 ml of any cyanobacterial culture, grown in BG11 medium was centrifuged at 4000 x g for 10 min. The pellet was resuspended in 500 µl of TES buffer and incubated for 2 hours at 4 °C. About 5 mg/ml lysozyme were added and the mixture was further incubated for 1 hour at 37 °C. 10 µl of 20 mg/ml Proteinase K, 0.05 M EDTA and SDS to a final concentration of 2.5 % were added, followed by incubation over night at 37 °C. The DNA preparation mixture was extracted twice with phenol/ chloroform/ isoamylalkohol (25:24:1), followed by chloroform/ isoamylalkohol (24:1). DNA was precipitated within 30 min using isopropanol. The pellet was washed again with 80 % ethanol, dried in vacuo, resuspended in 50 µl TE buffer and incubated for 5 min at 55 °C. Finally 1 µl of RNase A/T1 Mix was added and the DNA was incubated at 37 °C for 1 hour.

5.5 Plasmid and fosmid extraction

Plasmids isolation was carried out using a QIAprep Spin Miniprep Kit. Fosmid DNA was purified using alkaline lysis with buffer P1, P2 and P3 similar to the method previously described [Morelle, 1989].

5.6 Quantification of DNA

DNA concentration was measured using the NanoDrop ND-1000 as recommended by the manufacturer.

5.7 DNA agarose gel extraction

DNA was purified from agarose gels using the JETSORB Gel Extraction Kit following the manufacturer's instruction.

5.8 Routine cloning procedure

Qiagen PCR cloning kit has been used for direct cloning of PCR products, amplified by *Taq* DNA polymerase, via TA cloning strategy. Purification of PCR products prior to cloning has been achieved by the use of GeneJET PCR Purification Kit.

5.9 Ligations

Ligations were conducted with T4 DNA Ligase and the appropriate buffer from Fermentas as indicated in the enzymes manual. Commonly, ligations were made overnight in the refrigerator at approximately 8 °C.

5.10 Restriction digests

Restriction digests were practiced under optimum conditions for each enzyme in single and double digests with enzymes purchased from either Fermentas or New England Biolabs.

5.11 DNA agarose gel electrophoresis

Conventional agarose gel electrophoresis was carried out with 1 % agarose in 1x TAE. Gels were run between 80-120 V. For some analysis a concentration of 0.7 % agarose was used. Ethidium bromide with a final concentration of 0.05 mg/ml was added to the gels. DNA samples were mixed with 6x loading dye from Fermentas. A Lambda-*Pst*I digest was used as a size standard.

5.12 Primers

Primers were ordered from Sigma-Genosys (Munich, Germany) or Eurofins MWG Operon (Ebersberg, Germany). A list of primers is given in the appendix. Restriction sites and other nonannealing parts of a primer are shown in lower case.

5.13 Polymerase chain reaction

PCR was performed in a T3000 thermocycler from Biometra. Tubes contained 20-50 µl reaction cocktail, including *Taq* DNA Polymerase (Qiagen), *Thermus aquaticus* DNA polymerase [Desai and Pfaffle, 1995], *Phusion* (Thermo Scientific) or *Pfu* DNA Polymerase (Qiagen) and their corresponding buffers system. dNTPs were purchased from Fermentas. Genomic or plasmid DNA was used as a template. The amplification for most types of PCRs is explained in the table below.

Table 5-2: Standard PCR amplification conditions

cycles	temperature in °C	time in min	relevance
1	95	3	initial denaturation
	95	0.5	denaturation
30	primer dependent	0.5-1	annealing
	72	1-7 (1 kb/min)	extension
1	72	10	final extension
1	15	∞	storage

5.14 Sequencing

Sequencing was carried out commercially in the laboratory of Dr. Martin Meixner (SMOLBIO) at Humboldt University of Berlin, Germany.

5.15 DNA and protein computer analysis

DNA sequence data was analyzed using BIOEDIT version 7.0.9.0 and the programs of NCBI BLAST [Altschul, et al., 1990]. Protein sequences for alignments were retrieved from the NCBI data base. Protein sequences were analyzed using ExPASy Proteomics server (http://expasy.org/). Multiple sequence alignments were accomplished by CLUSTALW (http://www.genome.jp/tools/clustalw/). The presence of signal peptides was analyzed by the following program: http://www.cbs.dtu.dk/services/SignalP. Subcellular localization of peptides was predicted by http://www.psort.org/.

5.16 Chemical structure and plasmid map design

Chemical structures were drawn using Isis Draw 2.4 and Chem Sketch 12.01. Plasmid maps were designed using SimVector 4.2. Final adaptations were made by Adobe Illustrator CS2.

5.17 Preparation of electro competent *E. coli*

A starter culture of *E. coli* was grown in LB overnight at 37°C under vigorous agitation. 30 µl of this culture were transferred to a fresh tube containing 1.4 ml LB. Cells were grown for 3 hours, removed from the incubator and chilled on ice. Cells were centrifuged for 30 s at 11000 rpm in a microcentrifuge and washed twice with 1 ml sterile water. 20-30 µl of supernatant were left with the pellet to resuspend the cells.

5.18 Preparation of chemically competent *E. coli*

400 ml LB were inoculated with 1 ml overnight culture of *E. coli* and incubated at 37 °C until OD_{600}=0.6. Cells were chilled on ice and centrifuged at 3000 x g. The pellet was resuspended in 100 ml of sterile $CaCl_2$-solution and centrifuged as before. Resuspension and centrifugation were repeated, the supernatant was removed and the cells were resuspended in 10 ml $CaCl_2$-solution. Cells were immediately used for transformation or aliquots of 100 µl were transferred to liquid nitrogen and finally kept at -80 °C.

5.19 Transformation of *E. coli*

Electrotransformation was performed using a Bio-Rad Gene Pulser. 1-2 µl DNA were mixed with 50 µl cells, incubated briefly on ice, transferred to a chilled electroporation cuvette and pulsed at 1.8 kV, 200 Ω and 25 µF. If transformation was performed using chemically competent *E. coli*, 10-100 µl cells were mixed with 1-20 µl of any plasmid, briefly chilled on ice, followed by heat shock for 30-75 s using a water bath at 42°C. 200-1000 µl LB were immediately added and the cells were kept for 1 hour recovery at 37°C before spreading on selective plates. Sometimes 40 µl X-Gal (20 mg/ml) and 40 µl IPTG (0.1 M) were added to 25 ml solid LB to obtain blue and white clones for selection.

5.20 Lysogenization of *E. coli*

The Lambda DE3 Lysogenization kit (Merck) has been used to integrate Lambda DE3 prophage into the *E. coli* Epi300T1R genome following the manufacturer's instructions.

5.21 Construction and screening of a fosmid library

The fosmid library has been constructed using the CopyControl Fosmid Library Production Kit and genomic DNA of *Microcystis aeruginosa* Nies843. As minor change from the manufacturer's instructions, T4 DNA ligase from Fermentas has been used to ligate pCC1FOS with 30-40 kb of gDNA.

Screening of the library was conducted by a PCR-based strategy using the *Taq* DNA polymerase (Qiagen) and the primer pair mdnBfw and mdnBrv. DNA was amplified under the following conditions: 94° C for 3 min, 94°C for 30 s, 55°C for 1 min, 72°C for 2 min, 34 cycles, 72°C for 10 min. Clones were first analyzed in pools of 12 overnight cultures of *E. coli* EPI300T1R carrying fosmids. PCR pools were made using 1 µl from each of 12 overnight cultures. 2.5 µl of these pools were used in each pool PCR. If primers amplified a product in a pool PCR, each of the corresponding individual overnight culture was analyzed separately using 2.5 µl culture as a template in a single-clone PCR. Fosmids were

sequenced from both ends with primer pair FW-pCC1/pEpiFOS and RV-pCC1/pEpiFOS. Sequence data were analyzed using NCBI BLAST [Altschul, et al., 1990].

5.22 Heterologous expression of microviridin from fosmids

250 ml of LB containing 12.5 mg/l chloramphenicol were inoculated with 25 ml of an overnight culture of *E. coli* Epi300T1R carrying a fosmid. Cultures were immediately induced to a high copy number using Copy Control Induction Solution as recommended by the manufacturer. Cells were commonly harvested after 5 hours.

5.23 Fosmid knockouts using Lambda Red system

Fosmid knockouts were created by the Counter-Selection BAC Modification Kit from Gene Bridges. The genes *mdnA*, *mdnB*, *mdnC*, *mdnD* and *mdnE* were knocked out according to the manual. Primers are listed in the appendix. Epi300T1R served as the host strain.

5.24 Duet vector system

The genes *mdnA*, *mdnB*, *mdnC*, *mdnD* and *mdnE* from *Microcystis aeruginosa* Nies298 were amplified by *Taq* DNA polymerase (Qiagen) with primers P91 to P100 and first cloned into pDrive cloning vector (Qiagen). Primer sequences are listed in the appendix. Gene products were excised with appropriate restriction enzymes, as shown in the table below, to be cloned in frame into the compatible Duet-1 vectors (Merck) with individual cloning sites and T7 promoters for every single gene. Single vectors or combinations of them were expressed in *E. coli* BL21 cells, as described in paragraph 5.41. Test protein expression was conducted as further explained in paragraph 5.42. Test protein purification was made from 50-100 ml *E. coli* cultures using Ni-NTA agarose or S-protein agarose, as described in paragraphs 5.43 and 5.44.

Table 5-3: Cloning of mdn genes into Duet vectors

Duet vector	cloned gene	restriction site
pETDuet-1	mdnA, mdnB	BamHI/EcoRI,
	mdnC	NdeI/BglII
pACYCDuet-1	mdnB	BamHI/EcoRI or NdeI/BglII
	mdnC	NdeI/BglII
pCOLADuet-1	mdnD	BamHI/EcoRI
	mdnE	NdeI/XhoI

5.25 Construction of *mdnE* partial deletion mutants in pCOLADuet-1

A PCR based strategy, using primer pairs P99-P149 and P100-P151 was applied to amplify *mdnE* from *Microcystis aeruginosa* Nies298 lacking 138 bases from the N-terminus or a 108 bases of sequence from the C-terminus. Amplified genes were cloned in frame into pCOLADuet-1-mdnD to obtain the following plasmids: pCOLADuet-1-mdnD/$E_{deletion3}$ and pCOLADuet-1-mdnD/$E_{deletion4}$. These constructs were transformed to *E. coli* Epi300T1R carrying the MRC-derived fosmid. The cultures were expressed and analyzed by HPLC.

5.26 Co-expression of fosmid and plasmid

The co-expression of a fosmid and a plasmid was achieved by transferring the plasmid via transformation into the fosmid host strain EPI300T1R. Cells were grown under standard conditions in the presence of the appropriate antibiotics to maintain both DNA elements.

5.27 Construction of a microviridin expression platform in pDrive

The microviridin biosynthetic gene cluster has been amplified from genomic DNA of *Microcystis aeruginosa* Nies843 using *Taq* DNA polymerase (Qiagen). Primer pair P166-P167 amplified the genes *mdnABCDE* with a short promoter region of 169 bases. The genes *mdnABCDE* including a long promoter region of 714 bases were amplified using primers P173-P167. Primer pair P173-P97 amplified the microviridin cassette from *mdnABCD*, lacking *mdnE*, but carrying the same 714 bp long promoter region, as described before. All PCR fragments were agarose gel-purified using JETSORB Gel Extraction Kit and ligated into pDrive, as described in 5.8.

5.28 Heterologous expression of microviridins from pDrive

400 ml LB supplemented with 50 mg/l ampicillin were inoculated with 30-40 ml overnight culture of *E. coli* Top10 or XL1 Blue cells carrying any microviridin expression platform. Cultures were grown under standard conditions, commonly for 5 hours, as described in 5.2.

5.29 Isolation of microviridins for HPLC analysis

Commonly, 400 ml of *E. coli* cultures were harvested by centrifugation at 4 °C, 5000 x g for 10 min. The pelleted cells were washed once with Tris-HCl, pH 7.4 and resuspended in 5 ml deionized water. Cell lysis was achieved by sonication for 90 s. The samples were centrifuged again and purified on C18-Sep-Pak cartridges (Waters). Cartridges were equilibrated with 2-5 ml of 100 % methanol and washed with 2-5 ml of water. Samples were transferred to the columns. Before elution with 2 ml 100 % methanol, cartridges were

washed once with 2 ml 5 % methanol. Eluted compounds were dried in vacuo, resuspended in 200-400 µl 50 % methanol and again filtered over Acrodisc 4 mm syringe filters (Pall).

5.30 HPLC analysis

HPLC was performed using a Shimadzu C18 RP-HPLC with a Symmetrie Shield Sentry Guart column (3.5 µm, 3.9x20 mm), a SymmetrieShield RP18 (3.5 µm, 4.6x100 mm) column and a UV/VIS detector. Water-0.05 % TFA as solvent A and acetonitrile-0.05 % TFA as solvent B were used as a mobile phase with gradient conditions as follows: Flow rate 1 ml/min, 1 min of loading to 20 % solvent B, a linear gradient to 52 % solvent B within 30 min, followed by a linear gradient to 100 % solvent B in 1 min enduring for 2 more minutes, before returning to 20 % solvent B in 3 min, holding for 5 minutes. The injection volume was 55 µl per run, if not indicated otherwise.

5.31 Standards for HPLC analysis

HPLC purified microviridin B from *Microcystis aeruginosa* Nies298 and microviridin L from *Microcystis aeruginosa* Nies843 were used as standards. Both peptides were isolated from a recombinant source (*E. coli*).

5.32 Mass spectrometry

Structure elucidation off all microviridins has been carried out in cooperation with Dr. Keishi Ishida from the Leibniz Institute for Natural Product Research and Infection Biology e.V. Hans-Knöll-Institute (HKI) in Jena by mass spectrometry. MALDI-TOF MS and MALDI-TOF/ TOF MS (PSD) were performed with a Bruker Ultraflex from Bruker Daltonics. Data were analyzed by flexAnalysis and illustrated using the Adobe illustrator software. Taniguchi et al. 2005

5.33 Protease inhibition assays

Protease inhibition assays were made in cooperation with Dr. Keishi Ishida (HKI Jena), as previously described [Shin, et al., 1996]. Additionally subtilisin has been included and the assay has been performed by the modified method [Taniguchi, et al., 2005].

5.34 Microviridin quantification

Microviridins were quantified by HPLC in triple biological replicates and calculated based on the peak height. Arrow bars indicate the standard deviation.

5.35 Quik change II XL mutagenesis

The Quik change XLII mutagenesis kit (Agilent) has been used to mutate any microviridin expression platform in pDrive as recommended by the manufacturer's instruction with the following PCR conditions for all primer combinations: 95 °C for 1 min, 18 x (95 °C for 50 s, 60 °C for 50 s), 68 °C for 10 min 30 s, 68 °C for 7 min, cooled to 15 °C. A list of primers is given in the appendix.

5.36 Factor Xa Protease treatment of microviridins

Digests with Factor Xa Protease were conducted similar as described in "The QIAexpressionist, June 2003" from Qiagen. A Factor Xa Protease cleavage site –IEGRI- was inserted at the C-terminus of the leader sequence (30-PSPEPTIEGRIYGGTFKYPSDWEDY) in the microviridin platform vector using the Quik change XLII site-directed mutagenesis kit, as described in paragraph 5.35. Isolated microviridin extracts were digested using 95 µl Factor Xa reaction buffer and 5 µl enzyme at room temperature or 37 °C for 16 h. Digests were analyzed by HPLC and mass spectrometry.

5.37 Hydroxylamine hydrochloride cleavage of microviridins

Digests with hydroxylamine hydrochloride were performed as previously described [Crimmins, et al., 2001]. A hydroxylamine cleavage site was constructed by the exchange of threonine (Y) against asparagine (N) in the microviridin coding sequence of MdnA (YGGTFKYPSDWEDY→NGGTFKYPSDWEDY) in the microviridin platform vector using the Quik change XLII site-directed mutagenesis kit, as described in paragraph 5.35. Isolated microviridin extracts were cleaved and analyzed by HPLC and mass spectrometry.

5.38 Trypsin cleavage of microviridins

An arginine (R) residue, as a trypsin cleavage site, was introduced at the C-terminus of the microviridin leader peptide (30–PSPEPTRYGGTFKYPSDWEDY) in the small microviridin expression platform using Quik change mutagenesis. Purified microviridins from this construct were treated with trypsin under various conditions. First of all, isolated microviridin extracts were incubated with 45-49 µl of 25 mM NH_4HCO_3 and 1-5 µl sequencing grade trypsin (1 µg/µl) for 1 or 2 hours at 37°C or 50 °C. Secondly, microviridins were digested following [Riviere and Tempst, 2001]. Microviridin extracts were resuspended in 25 mM NH_4HCO_3 containing 1 % SDS, heated to 95 °C for 10 min, before again 100 µl NH_4HCO_3 and 5 µl trypsin were added. The reaction was incubated for 1 hour at 50 °C. SDS was precipitated using guanidine-HCl and removed by centrifugation.

And thirdly, microviridin extracts were digested using 80 µl 25 mM NH_4HCO_3, 20 µl acetonitrile and 5 µl of trypsin for 1 hour at 50 °C. All digests were analyzed by HPLC and mass spectrometry.

5.39 Microviridin localization in *Microcystis aeruginosa*

300 ml of *Microcystis aeruginosa* Nies843 (OD_{750}=0.342) were harvested by centrifugation at 5000 x g for 10 min at 4 °C. Periplasmic proteins were isolated similar to the method described by [Fulda, et al., 1999] using 10 M Tris-HCl (pH 7.6) and plasmolysis buffer for cyanobacteria with 2 mM NaCl. The osmotic shock supernatant, representing the periplasmic fraction, was collected and the remaining cell pellet was washed once with water. 1 ml of water and the same volume of glass beads were added to the pellet and the mixture was placed in liquid nitrogen. Cells were thawed in a water bath at 42 °C. Cells were disrupted using a Retsch mixer mill for 10 min. The freeze, thaw and disruption procedure was repeated 3 times. After centrifugation the supernatant, containing the cytoplasmic fraction, was collected and the pellet was washed again with water, before 4 ml of methanol were added to extract the membrane fraction. Total cell extracts were made from 300 ml of an identical culture using the same procedure as described for the cytosolic fraction. Microviridins from isolated membrane fraction together with cytoplasmic, periplasmic and total extract were purified on C18 Sep-Pak cartridges, as described in paragraph 5.29. The BG11 growth medium has been analyzed for the presence of microviridins by the same C18 Sep-Pak purification procedure.

5.40 Microviridin localization in *E. coli* carrying the small expression platform

400 ml of *E. coli* Top10, carrying a microviridin expression platform vector as well as plain Top10 cells were grown under standard conditions for 5-7 hours. Cells were harvested by centrifugation at 4000 x g for 20 min. The pellet was resuspended in Tris-HCl-30 % sucrose (pH 8.0) at 80 ml per gram wet weight. Cells were kept on ice and EDTA was added to a final concentration of 1 mM. Cells rested for 10 min on ice under slightly agitation, followed by centrifugation for 20 min, 8000 x g at 4 °C. The pellet was resupended in the same volume of ice cold 5 mM $MgSO_4$ and stirred for 10 min on ice. The cell suspension was again centrifuged as before. The resulting supernatant was the osmotic shock fluid containing the periplasmic proteins. This fraction was collected and the pellet was washed again with 1 M Tris-HCl pH 7.4. 5 ml of deionized water were added to resuspend the pellet. Cell lysis was achieved by sonication for 90 s. Cells were centrifuged again and the supernatant was collected, representing the cytoplasmic protein fraction. The remaining pellet, representing the membrane fraction, was washed once with water. Resuspension was achieved by the addition of 5 ml methanol and a pulse of sonication. The supernatant

was collected. For the comparison of subcellular localization of microviridin in periplasmic, cytoplasmic and membrane extract with the total cell extract, 400 ml of *E. coli* cells were centrifuged at 4000 x g for 20 min. The pellet was washed once with Tris-HCl pH 7.4, centrifuged and resuspended in 5 ml deionized water. Sonication was used to achieve complete cell lyses. Centrifugation was repeated and comprised the total cell extract. Microviridins from the total extract and from all supernatant fractions were purified for HPLC analysis, as described in paragraph 5.29. In some cases PMSF was added to the lyses' steps to prevent protein degradation.

5.41 Protein over-expression in *E. coli*

Cultures were grown in LB medium, containing the appropriate antibiotics, at 18-37 °C and 240 rpm until OD_{600} = 0.6-0.8. Cells were induced with IPTG to a final concentration of 0.4-1 mM and further grown for 3-4 hours. Cultures were centrifuged for 10 min at 5000 x g and the pellets were washed with Tris-HCl pH 7.5. Sometimes pellets were kept at -20 °C overnight.

5.42 Preparation of *E. coli* lysates under denaturing conditions

Cell pellets from 2 ml cultures, grown similar to the procedure described under 5.41, were resuspended in 500 µl buffer B and the same volume of glass beads. Pellets were disrupted using a Retsch mixer mill for 10 min. The resulting protein extract was mixed with SDS-loading dye, heated to 95°C for 5 min and run on an SDS-polyacrylamid gel.

5.43 Protein purification using S-Agarose

Proteins were purified under native conditions using S-protein Agarose, as recommended by Merck. 3 M $MgCl_2$ was used as an eluent. S-protein agarose was recycled several times. Aliquots of each fraction were mixed with SDS-loading dye, heated to 95°C for 5 min and run on an SDS-polyacrylamid gel.

5.44 Protein purification using Ni-NTA Agarose

His-tag fusion proteins from pETDuet vectors were purified under native conditions using Ni-NTA agarose, as described by the manufacturer (Qiagen). Lysis buffer contained 10 mM imidazol, wash and elution buffer contained 30 and 250 mM imidazol, respectively. Aliquots of each fraction were mixed with SDS-loading dye, heated to 95°C for 5 min and run on an SDS-polyacrylamid gel.

5.45 Periplasmic protein purification using Ni-NTA Agarose

Periplasmic proteins were over-expressed in *E. coli* and purified under native conditions using Ni-NTA agarose (Qiagen), following the instructions given in "The QIAexpressionist, June 2003" from Qiagen. Dialysis was performed using Visking dialysis tubing 20/32 and 5 l lysis buffer, containing 10 mM imidazole. Wash and elution buffer contained 30 and 250 mM imidazol, respectively. Aliquots of each fraction were mixed with SDS-loading dye, heated to 95°C for 5 min and run on an SDS-polyacrylamid gel.

5.46 Bradford protein assay

The protein concentration was measured at 595 nm by the method of Bradford [Bradford, 1976] using Protein Assay solution from Bio-Rad.

5.47 SDS-PAGE

Size separation of proteins was performed as previously described by [Laemmli, 1970] on gels in the size of 7x8 cm using the Mini-Protean system from Bio-Rad. Stacking gels, containing 4 % and separating gels containing 12.5 % acrylamid, respectively, were prepared using Rotiphorese Gel30 (37.5:1), stacking (pH 6.8) and separating gel buffer (pH 8.8), TEMED, 10 % APS and H_2O. Protein extracts were mixed with 5x SDS-loading dye containing 2-mercaptoethanol and heated to 95 °C for 5 min. Samples were transferred to the gel and PageRuler Prestained Protein Ladder was additionally load as a size standard. Gels were run under following conditions: 20 V for 20 min, followed by 200 V for 35-45 min. Subsequently gels were washed 3 times for 5 min with water, before they were incubated for 1 h with Gel-Code Blue Safe Protein stain. Afterwards gels were destained with water within 1 hour by addition of a Kimwipe tissue or overnight.

5.48 Western blot analysis and immunodetection

SDS-polyacrylamid gels, stained with Gel-Code Blue Safe Protein stain were 3 times alternately destained with 50 % acetonitrile for 20 min and rehydrated in water for 20 min, incubated for 30 min in 2 % SDS followed by incubation for 30 min in SDS running buffer. Proteins were transferred at 380 mA for 1 h to a Hybond-C membrane (GE Healthcare) using a tank blotter (Hoefer) filled with 1x transfer buffer. Afterwards membranes were removed from the blotting sandwich and blocked with 5 % skim milk powder in TBS-T or PBS-T for 1 hour at RT. Membranes were incubated with the appropriate primary and if necessary secondary antibody. Membranes were washed 3 times for 5 min in TBS-T or PBS-T prior to the addition of any secondary antibody and as a final washing before visualization,

which was performed using SuperSignal West Pico Chemiluminescent Substrate kit (Pierce) and a Lumi-Imager (Boehringer). A list of antibodies and their incubation conditions is given below.

Table 5-4: Incubation conditions for antibodies

antibody	incubation time in min	incubation temperature in °C	buffer
Anti-MdnB (rabbit)	60	RT	TBS-T
Anti-rabbit IgG horseradish peroxidase conjugate, produced in goat	60	RT	TBS-T
Monoclonal Anti-poly histidine antibody produced in mouse	60	RT	PBS-T
Mouse IgG, horseradish peroxidase linked whole antibody (sheep)	60	RT	PBS-T
s-protein HRP conjugate	30-45 min	RT	TBS-T

5.49 Protein concentration and purification using Amicon Ultra filters

Proteins were purified and concentrated using Amicon Ultra-0.5 mL Centrifugal Filters from Millipore with a MWCO of 30000 Da. Filters were centrifuged at 14000 x g for 10-20 min during concentration and at 1000 x g for 2 min during elution of proteins.

5.50 Dissolving synthesized peptides

Synthesized MvdE, from *Planktothrix agardhii* and MdnA(2)-triple PFFARFL mutated precursor from *Microcystis aeruginosa* Nies843 were dissolved in sterile PBS buffer pH 7.4 to a concentration of 20 µg/µl. MdnA-original Nies298 precursor was obtained as a dissolved peptide in Tris-HCl buffer pH 7-8. MdnA(1)-original Nies843 precursor was insoluble in PBS buffer pH 7.4. For SPR studies 3 µl of precursor were mixed with 50 µl DMSO and 450 µl sodium acetate buffer (pH 4.0) and further diluted as necessary. For *in vitro* enzyme assays the MdnA(1)-original precursor was mixed 1:2 with 1 % SDS and heated for 5 min at 95°C to mediate solubility.

5.51 Surface plasmon resonance

Surface plasmon resonance analysis was carried out with a BIACORE 2000 and a sensor chip CM5, using the control software 2.1 and evaluation software 3.0 (GE Healthcare). Synthesized MvdE, from *Planktothrix agardhii*, MdnA(1)-original and MdnA(2)-triple PFFARFL mutated precursor from *Microcystis aeruginosa* Nies843 in 10 mM sodium acetate

buffer (pH 4.0) were immobilized on the sensor chip via amine coupling at 410-494 response units per flow cell. HEPES buffer (pH 8.0) containing NaCl and KCl was used as a running buffer. MdnB and MdnC from *Microcystis aeruginosa* Nies298, each separately expressed and purified from pACYCDuet-1, were assessed for their activity towards the immobilized ligands. Both proteins with concentrations 0.15, 0.3, 0.6, 1.25, 2.5, 5.0, 10.0 and 20.0 µM were injected at a flow rate of 30 µl/min, followed by dissociation and regeneration of the sensor surface with HCl. BSA, POD, TusA, Mvn and ActM, proteins not expected to bind to the precursor molecules, were used as controls.

5.52 *In vitro* enzyme assay

Synthesized microviridin precursors MdnA(1)-original Nies843, MdnA-original Nies298 and MvdE from *Planktothrix* were dissolved, as described in 5.50. MdnB and MdnC from *Microcystis aeruginosa* Nies298 were separately expressed from pACYCDuet-1 using *E. coli* BL21 (DE3) cells, as described in paragraph 5.41. *In vitro* cyclization of MdnA was made similar as previously described [Philmus, et al., 2008] using up to twice as much protein MdnB, MdnC and $MdnE_{cytosolic}$. Aliquots were analyzed by C18 RP-HPLC under conditions described in paragraph 5.30 and verified by mass spectrometry. The cytosolic domain of MdnE ($MdnE_{cytosolic}$) was cloned into the *Nde*I/*Bgl*II site of pACYCDuet-1, expressed in BL21 (DE3), purified over S-protein agarose and kindly provided as a purified protein by Douglas Gatte-Picchi (University of Potsdam, group of Prof. Elke Dittmann).

6 Results

At the beginning of this study it was known that microviridins were produced through a ribosomal pathway biosynthesis, commonly in the presence of the genes *mdnABCDE*. Two ATP-grasp ligases, MdnB and MdnC, cross-link the microviridin precursor peptide MdnA in a strict order and establish two ω-ester followed by one ω-amide bond. Additionally the N-acetyltransferase MdnD was believed to modify microviridins posttranslationally, although a proof *in vivo* was still missing. At that time MdnE, the ABC-transporter, was assumed to participate in the peptide cleavage itself, maybe carrying a so far uncharacterized peptidase domain or being supportive in a different manner.

6.1 Microviridin L production and structure elucidation

A new microviridin gene cluster has been detected from the genome sequence of *Microcystis aeruginosa* Nies843 [Kaneko, et al., 2007]. To analyze these genes, heterologous expression was chosen. This strategy is a feasible alternative to fermentation process development and molecular manipulation of the native producer strain, as the cyanobacterium is slowly growing and additionally highly resistant to genetic engineering strategies. Genomic DNA of the cyanobacterial producer was isolated and fragments of 30-40 kb were directly ligated to the fosmid vector pCC1FOS. The primary library was made by infection of *E. coli* Epi300T1R. Plating and selection for chloramphenicol resistant clones revealed about 800 colonies, of which 5 were randomly picked and subjected to DNA isolation and restriction enzyme digestion. Thus, the average insert size of the library was determined to be in the range of 40-43 Kb (data not shown), which was later proven by sequence analysis and comparison to the available complete genome sequence of Nies843 [Kaneko, et al., 2007]. A total of 518 fosmid clones were screened by colony PCR to identify Fos303N843, carrying the genes *mdnABCDE* for microviridin L production (Figure 6-1, A). Heterologous expression of Fos303N843 next to a control fosmid, lacking the machinery for microviridin production, showed the following HPLC chromatogram (Figure 6-1, B). Peak fractionation and mass analysis showed the presence of several different tricyclic microviridin-like peptides which all differed, in correspondence to the amino acid sequence encoded by MdnA, in their length at the N-terminus, as given in (Figure 6-1, C). Four peaks were further investigated. Their structures were elucidated and named following the common nomenclature as microviridin L, L1, L2 and L3. As all identified microviridins from original producers so far consist of 13-14 amino acids (Figure 1-9) microviridin L and L1 were considered to be the completely processed peptides. A microviridin structure with highlighted two ω-ester and the secondary ω-amide bond is shown in Figure 6-1, C. The longer microviridin-like peptides were considered as

incompletely processed microviridins as a result from heterologous expression, and are further referred under this nomenclature. In *E. coli,* they appear in a large proportion compared to the correctly processed microviridins, which is completely different in the original producer, where only microviridin L and L1 can be detected by HPLC. Figure 6-2 shows an HPLC profile of a Nies843 cell extract compared to a microviridin L standard, previously isolated and purified from Fos303N843. None of the additional small peaks could be identified as a microviridin-like compound by mass spectrometry (data not shown).

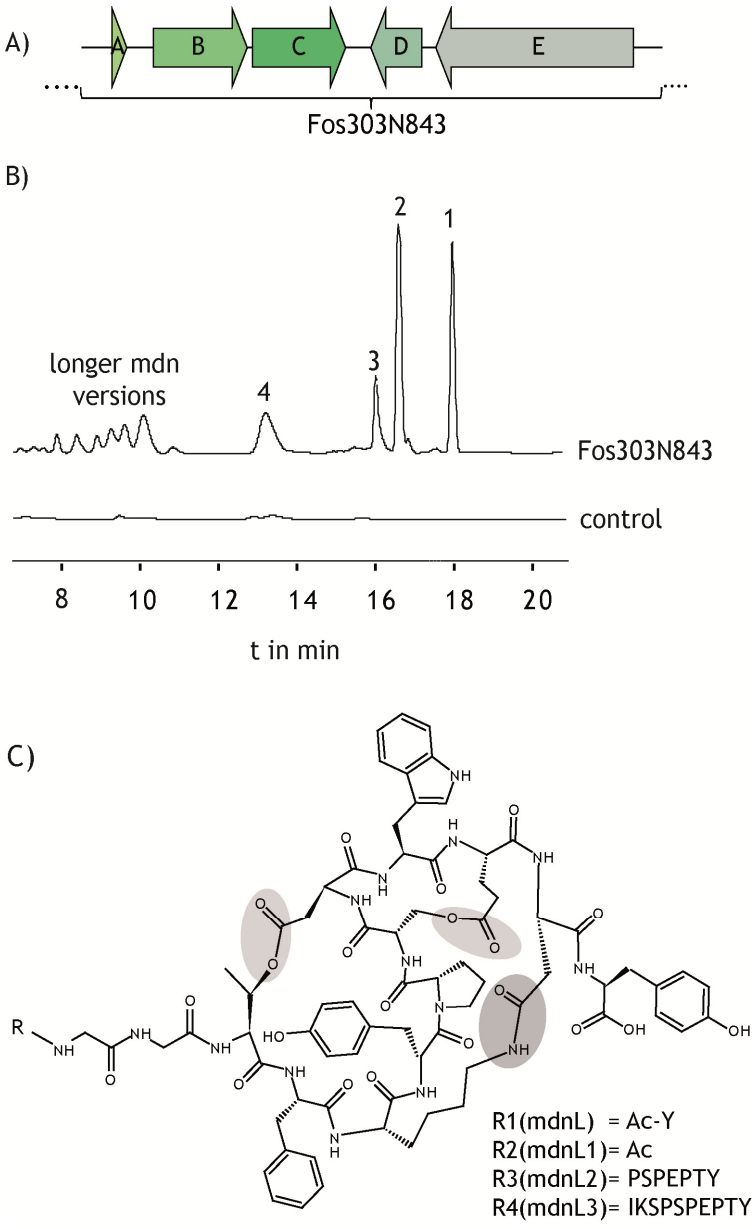

Figure 6-1: Heterologous expression of microviridin L from Fos303N843

A schematic illustration of Fos303N843 carrying the microviridin expression cassette is shown in A) Dots indicate the fosmid carries additional genes from Nies843, totally about 43 kbp. Microviridin L and its derivatives were expressed from Fos303N843 in *E. coli*, separated by RP-C18 chromatography and detected at 210 nm. The numbers indicate microviridin L (1), L1 (2), L2 (3), L3 (4) as given in B). The chemical structure of microviridin L and its derivatives L1, L2, L3 is illustrated in C). The two ω-ester bonds are highlighted in light gray; the ω-amide bond is shown in darker gray. Ac: acetyl

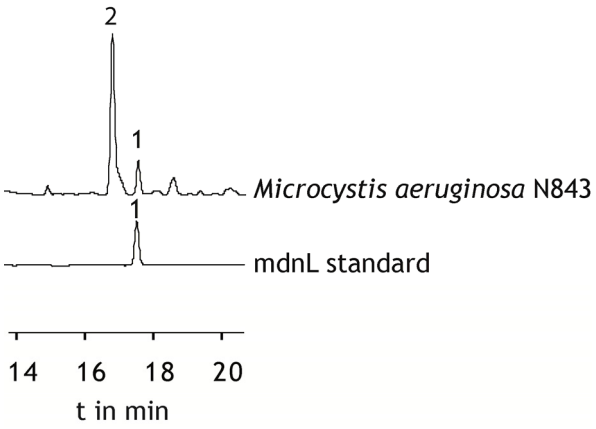

Figure 6-2: HPLC chromatogram of microviridin L production in *Microcystis aeruginosa* Nies843

Mdn was separated by RP-C18 chromatography and detected at 210 nm. The microviridin L standard was isolated from *E. coli* carrying Fos303N843. The numbers indicate microviridin L (1) and L1 (2).

6.2 Microviridin gene knockouts on the fosmid Fos303N843

To analyze the function of the *mdnABCDE* genes previously cloned into the fosmid backbone and to later replace certain genes from the cluster by mutated versions, the Red/ET based recombination system provided by the Counter-Selection BAC Modification Kit (Gene Bridges) was used to knockout every single gene by homologous recombination. An *rpsLneo* resistance cassette was inserted maintaining about 50 bases of N- and C-terminal sequence of every gene. The resulting mutants Fos303N843-Δ*mdnA*, -Δ*mdnB*, -Δ*mdnC*, -Δ*mdnD*, -Δ*mdnE* were first analyzed by PCR for the presence of the *rpsLneo* cassette (data not shown) and positive clones were further characterized by HPLC (Figure 6-3) and mass spectrometry (data not shown). The effect of Red/ET recombination is clearly visible, as the production of microviridin L was completely abolished when *mdnA* was knocked out. Similar results were obtained for Fos303N843-Δ*mdnC*, which was expected at that point and in correspondence to the results previously published [Philmus, et al., 2009]. As MdnC is the ester bond introducing enzyme, it catalyzes the first cyclization step in microviridin biosynthesis. MdnB can introduce the amide bond only once the ester bonds are established. Interestingly, Fos303N843-Δ*mdnB* showed a similar result as Fos303N843-Δ*mdnC*, as no peaks were detectable in the chromatogram although the ester ligase remained untouched. Mass spectrometry confirmed the presence of trace amounts of bicyclic microviridin L and L1 from the Fos303N843-Δ*mdnB* mutant (data not shown). The chemical structures of bicyclic microviridin L and L1 are shown in Figure 6-4.

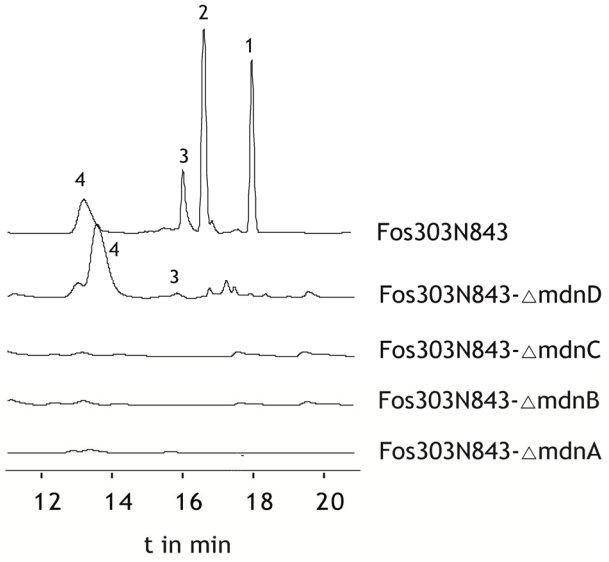

Figure 6-3: HPLC chromatogram of Red/ET mediated recombination in Fos303N843

Microviridins were separated by RP-C18 chromatography and detected at 210 nm. Original Fos303N843 is shown together with the corresponding knockout mutants of *mdnABCD* genes generated by Red/ET recombination in the fosmid. The numbers indicate microviridin L (1), L1 (2), L2 (3), L3 (4).

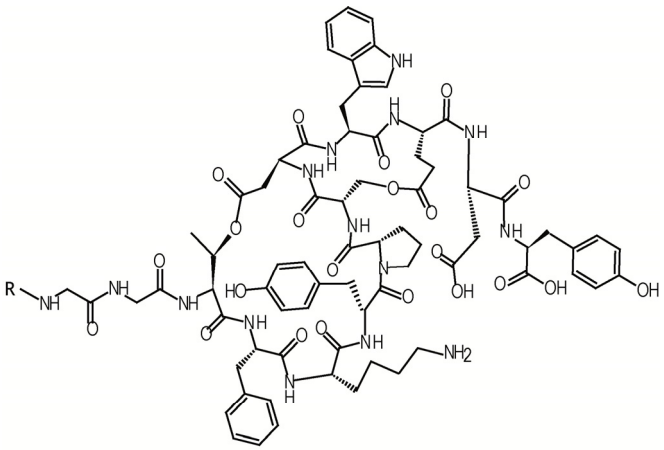

Figure 6-4: Chemical structure of bicyclic microviridin L and L1

Ac: acetyl

The knockout of *mdnD* was accompanied by the loss of the two acetylated microviridins, L and L1, which was the first indication *in vivo* for a function of MdnD in the acetylation of microviridin L and L1. *In vitro* the function of MvdB, which is an ortholog of MdnD from *Planktothrix agardhii*, has been previously shown [Philmus, et al., 2008]. Furthermore, nonacetylated forms of microviridin L and L1 were detected by mass spectrometry. These variants are not displayed in the HPLC profile, as they eluted together with the incompletely processed microviridins, which led to very poor separation of the peaks.

Several attempts were made to knockout *mdnE*, because MdnE was so far assumed to be the most probable candidate for peptide processing and cleavage of microviridins. This assumption was based on previously published data [Ziemert, et al., 2008]. There it was shown, that in the absence of *mdnD* and *mdnE* only incompletely processed microviridins were produced from an MRC-derived fosmid carrying exclusively *mdnABC* genes. Unfortunately, no reliable knockout of *mdnE* was obtained in this study. All candidates seemed to be heterozygous. The regions of homology at the end of the linear fragment probably annealed to more than the one site considered for recombination. Internal sequencing of fosmids starting from the inserted resistance cassette into adjacent regions was challenging. Restriction analysis was tried, but did not yield any clarifying result. Maybe the cells contained more than one fosmid at the time.

The replacement of the inserted *rpsLneo* cassette by a nonselectable DNA, meaning a mutated version of the knocked out microviridin genes *mdnA* to *D*, was never achieved.

Several attempts were made to complement the fosmid knockouts using copies of *mdnA*, *mdnB* and *mdnC* expressed from pET15b, pET40b(+) or pDrive vector, which were transferred into the Epi300T1R strain carrying a knockout fosmid. The microviridin expression at different incubation times, temperatures and in presence and absence of induction solution for fosmid and plasmid was analyzed by HPLC but microviridin biosynthesis has never been reactivated by this strategy (data not shown).

Thus, the fosmid system was not useful as a background to analyze mutations in the microviridin expression cassette.

6.3 Duet vector system

Another microviridin expression system was necessary to be established for further mutational analysis of the microviridin gene cluster. The Duet vector system (Merck) was previously shown to be suitable for the expression of the ribosomally synthesized patellamides [Donia, et al., 2006]. In a similar approach 3 compatible expression vectors with independent T7 promoter sites were chosen to express the *Microcystis aeruginosa* Nies298 genes *mdnABCDE, mdnABCD, mdnABCE* and *mdnABDE* as a control in *E. coli* BL21 cells. The genes of Nies298 were chosen instead of Nies843, because at that point it was believed that Nies298 tended to result in higher microviridin expression levels. The genes were amplified by PCR using primers P91 to P100 and cloned into pDrive, before they were excised with the appropriate restriction enzymes and cloned in frame into the Duet-1-vector backbones. Multiple clones of the Duet-1 vectors were cultured under varying conditions with different temperatures, IPTG concentrations and incubation times. HPLC

analysis, including Nies298 microviridin B standard, revealed no detectable microviridin-like peptides from any vector combination tried. As microviridin expression from fosmids was successfully shown in the Epi300T1R library host strain, the ΛDE3 prophage was integrated into the genome of this strain to express target genes cloned in T7 expression vectors, like the Duet-1 vectors are. Duet-1 vectors were transferred to Epi300T1R (ΛDE3) and analyzed as described before, but again no microviridin expression was detected. Thus, the use of BL21 cells was excluded to be the reason for the lack of expression.

When protein expression was inspected using Western blot analysis and immunodetection from total cell extracts of the Duet-1-vectors, positive results were exclusively obtained for MdnC (data not shown). It was assumed, the linear peptide MdnA was rapidly degraded and thus did not give any signals. Later during this work, MdnB has been expressed, but in very low concentration that made detection from small sample volumes, even as a purified protein, impossible. Very low expression of MdnD and maybe also MdnE was later shown in co-expression studies from fosmid and Duet vectors, as described in the next chapters.

6.4 Co-expression of fosmid and Duet vectors

In parallel to the expression studies of microviridin from the Duet vector system, pCOLADuet-1-mdnD, pCOLADuet-1-mdnD-E and pCOLADuet-1-mdnE were chosen to be expressed simultaneously with the MRC-derived fosmid carrying *mdnABC* genes for microviridin J production. The MRC-derived fosmid was reported to produce cyclized but incompletely processed microviridins [Ziemert, et al., 2008]. Indeed, the external addition of *mdnD* and *DE* on pCOLADuet-1 resulted in the production of comparatively very little amounts of correctly processed and cyclized microviridin J compared to the incompletly processed proportion. Microviridin J production was verified by mass spectrometry (data not shown). A comparison of the curves from MRC-derived fosmid-pCOLADuetmdnD-E with the MRC-derived fosmid plus pCOLADuet−1-mdnD showed a slightly higher level of microviridin production in presence of both enzymes, indicating they are both important for the correct biosynthesis of acetylated microviridin J. The low level of microviridin J production showed that MdnD was indeed expressed from the Duet vector and furthermore it was proven to be the acetylating enzyme in microviridin biosynthesis. The involvement of the ABC-transporter MdnE could not be clarified from this experimental set-up. It was also not clear, if MdnE was really expressed under these experimental conditions, which is why another co-expression experiment was started.

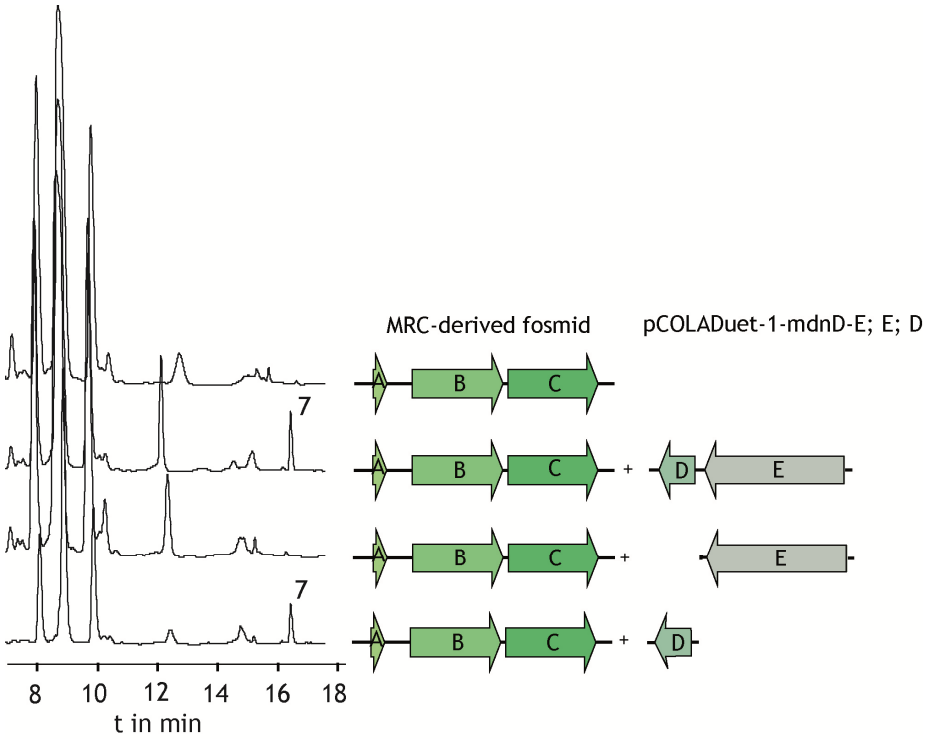

Figure 6-5: Co-expression experiments with MRC-derived fosmid and pCOLADuet-1 vector

MRC-derived fosmid carrying *mdnABC* and plasmid pCOLADuet-1 carrying *mdnDE*, *E* or *D* were expressed in *E. coli*. Microviridins were purified, separated by RP-C18 chromatography and detected at 210 nm. MRC-derived fosmid and pCOLADuet-1-vectors are shown schematically on the right, the HPLC profile is shown on the left. Microviridin J production is shown in (7).

Next, Fos303N843 carrying the genes *mdnABCDE* was transformed with pCOLADuet-1-mdnD-E, pCOLADuet-1-mdnE and pCOLADuet-1-mdnD and analyzed by HPLC and mass spectrometry (MS data not shown). The microviridin L and L1 production was faintly increased when extra copies of *mdnD* were added. The production was enhanced when *mdnDE* was co-expressed with Fos303N843, as shown in Figure 6-6. This phenomenon already appeared after short time expression for 3 hours at 37°C. Commonly, fosmid expression lasted for at least 5 hours at 37°C. These data again suggested that both the N-acetyltransferase MdnD and the ABC-transporter MdnE play a relevant role in microviridin biosynthesis. MdnD could be responsible for the stabilization of correctly processed microviridins through the introduction of an acetyl group at their N-terminus.

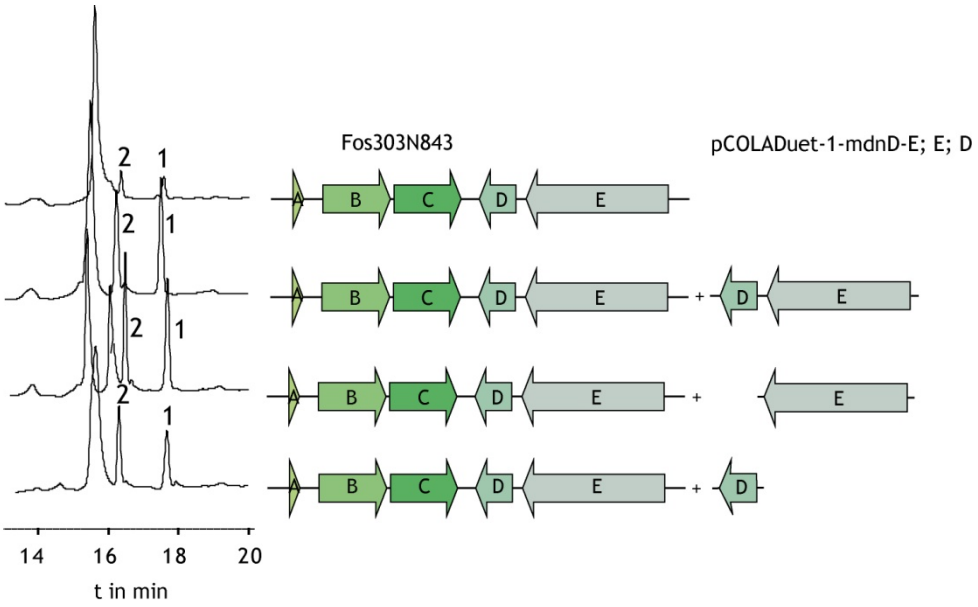

Figure 6-6: Co-expression experiments with Fos303N843 and pCOLADuet-1 vectors

Fos303N843 carrying *mdnABCDE* and plasmid pCOLADuet-1 carrying *mdnDE*, *E* or *D* were co-expressed in *E. coli*. Microviridins were purified, separated by RP-C18 chromatography and detected at 210 nm. Fos303N843 and pCOLADuet-1-vectors are shown schematically on the right, the HPLC profile is shown on the left. The numbers indicate microviridin L (1) and L1 (2).

To further analyze the ABC-transporter, a deletional PCR approach of *mdnE* was carried out. ABC-transporters from Gram-negative bacteria including the related cyanobacterium *Nostoc sp.* PCC7120 are known to carry N-terminal domains, which belong to the C39 protein family commonly involved in the processing of conserved leader peptides carrying a double glycine motif [Dirix, et al., 2004]. The microviridin leader peptides of *Microcystis aeruginosa* strains do not carry a double glycine motif and domain search analysis using BLASTP did not reveal any hits for protease domains at the N- or C-terminus of MdnE or in the close vicinity of the other proteins. It was assumed, for the processing of the cross-linked microviridin pre-stages to the mature compound, that a yet unidentified domain might be involved in the peptide cleavage. Therefore, N- and C-terminal deletions of *mdnE* were constructed and ligated to unmodified pCOLADuet-1-mdnD, resulting in pCOLADuet-1-mdnD/$E_{deletion3}$ and pCOLADuet-1-mdnD/$E_{deletion4}$. *E. coli* cells carrying the MRC-derived fosmid were transformed with one of the two pCOLADuet constructs at a time and analyzed by HPLC and mass spectrometry (MS data not shown). N- and C-terminal deletions in *mdnE* did not show an impact on the cleavage pattern of microviridins compared to the control with nonmutated pCOLADuet-1 vector. Microviridin J and the proportion of incompletely processed microviridins were produced in nearly the same amounts as before, which indicated there was no so far unknown N- and C-terminal peptidase domain located. But again, it has to be considered that it was not clear, if MdnE was really expressed from the Duet vector system under this experimental set-up.

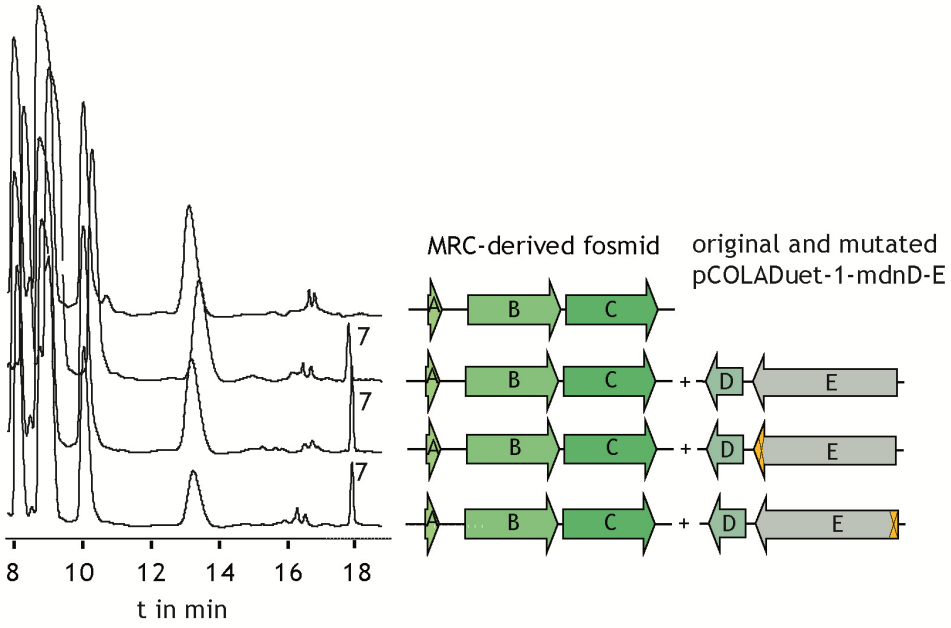

Figure 6-7: Co-expression experiments of the MRC-derived fosmid with mutated pCOLADuet-1 vectors

MRC-derived fosmid carrying *mdnABC*, pCOLADuet-1-mdnD-E original vector and the 2 pCOLADuet-1-mdnD-E vectors carrying N- and C-terminal deletions in *mdnE*, highlighted in orange, were co-expressed in *E. coli*. Microviridins were purified, separated by RP-C18 chromatography and detected at 210 nm. The MRC-derived fosmid and the pCOLADuet-1-vectors are shown schematically on the right, the HPLC profile is shown on the left. The production of microviridin J is shown in (7).

6.5 Construction of a small microviridin expression platform in pDrive

The Duet vector system was not applicable and the fosmid system previously used for the controlled and successful heterologous expression of microviridins was no longer suitable as the following work was aimed on the investigation of the microviridin gene cluster using site-directed mutagenesis. As fosmids are highly restrictive for point mutations, the small generic and available cloning vector pDrive (Qiagen) was chosen to serve as the backbone of the prospective small microviridin expression platform. The Nies843 microviridin gene cluster based on the genes *mdnABCDE* was amplified with primer pair P166-P167 including 169 bp of promoter region, representing the intergenic space between *mdnA* and the next annotated ORF MAE24120. The gel-purified PCR product was directly ligated into pDrive, transformed into *E. coli* XL1 Blue cells and selected on ampicillin, X-Gal, IPTG supplemented agar plates. Several white clones were first analyzed by restriction digests using *EcoR*I to cut out the insert. Potential candidates were sequenced to ensure the presence of the complete microviridin gene cluster. 8 clones carrying the desired insert were obtained. The plasmid was named pARW066-mdnABCDE-short (a plasmid map is given in the appendix). The candidates were analyzed by HPLC, but once more there was no evidence for microviridin expression. At the same time Katharina Makower (group of Prof. Dittmann, University of Potsdam) started to work on the transcriptional organization of the

microviridin gene cluster. Using Northern hybridization the transcriptional analysis of the gene clusters in *Microcystis* revealed the reason for the lack of microviridin expression from the pDrive construct. Two transcripts of about 450 and 600 bp were detected for *mdnA*. The larger one was missing from pARW066-mdnABCDE-short construct carrying only about 169 bp of promoter region [Makower, 2010]. With this data in hand, a second small microviridin expression platform was constructed, including again the genes *mdnABCDE* with about 714 bp of promoter region up to ORF MAE24140.

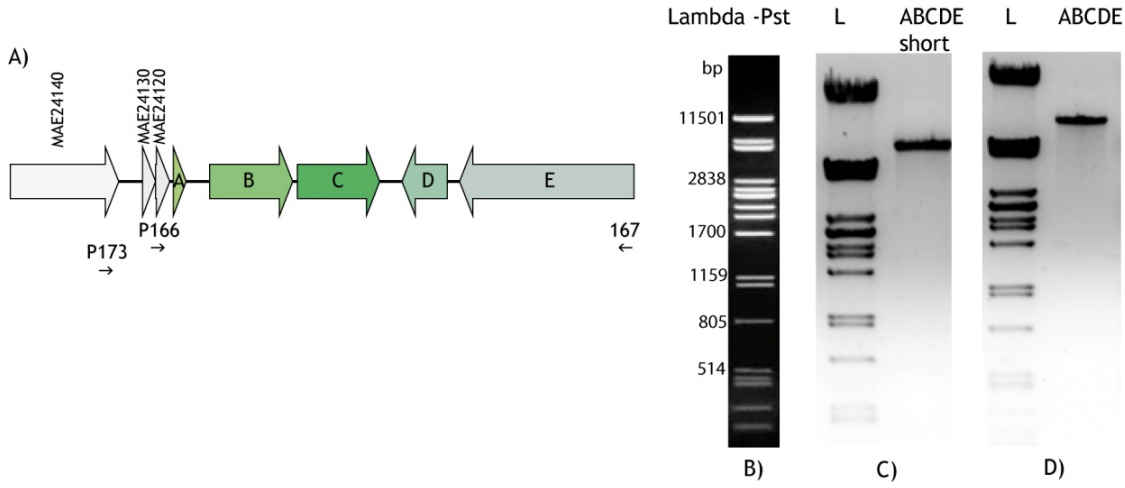

Figure 6-8: Agarose gels with the *mdnABCDE* PCR products

A schematic representation of the amplified mdn clusters is given in A). A Lambda-*Pst*I digest was used as a size standard, shown in B) and L. The *mdnABCDE* cassettes were amplified with primer pair P166-P167 to include a short promoter region as given in C). Primer pair P173-P167 amplified the genes *mdnABCDE* with a long promoter region as shown in D).

Sequencing was carried out to ensure that the whole cluster is present and *mdnA* is not mutated, as the cluster was amplified with a nonproofreading *Taq* polymerase. None of the proofreading enzymes as *Pfu* DNA polymerase (Qiagen) or *Phusion* High-Fidelity DNA Polymerase (Thermo Scientific) managed to amplify this gene cluster. The plasmid was named pARW071-mdnABCDE (a plasmid map is shown in the appendix). It was transformed into *E. coli* and analyzed by HPLC. In comparison to the fosmid Fos303N843 a similar peak pattern appeared.

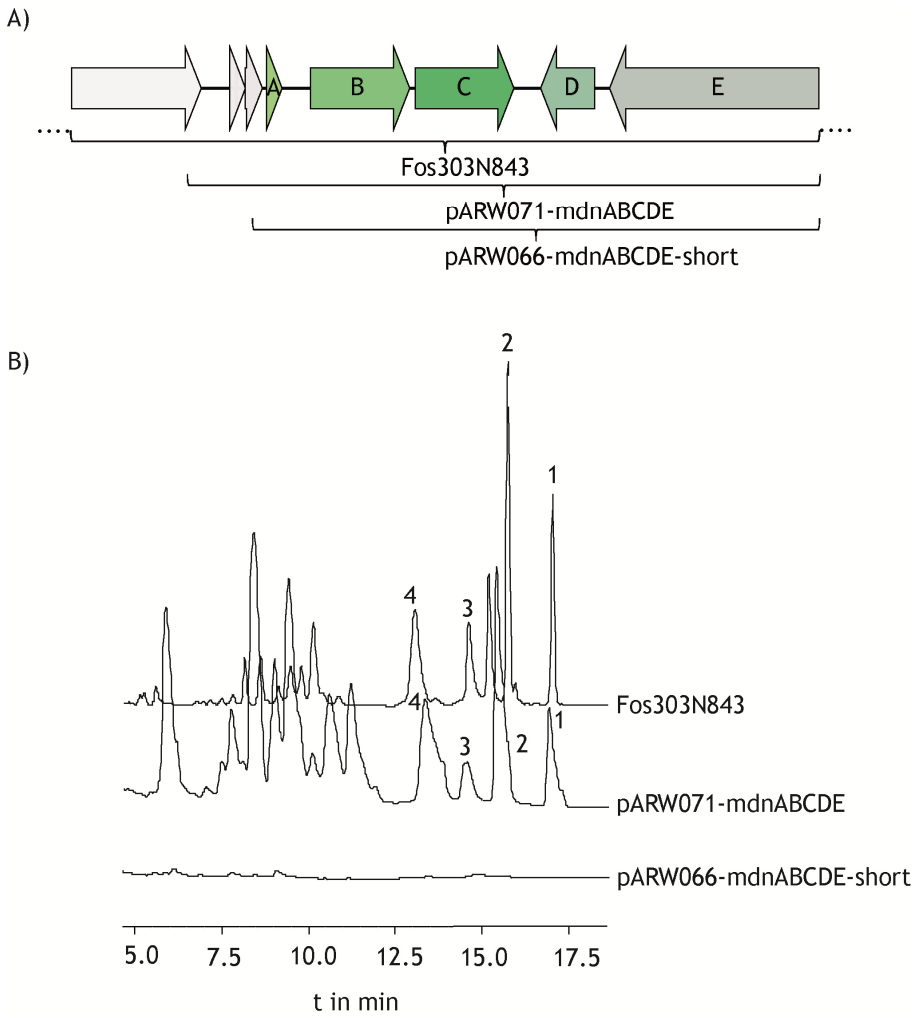

Figure 6-9: Microviridin expression from a the small pDrive based platform

A schematic representation of Nies843 microviridin gene cluster as it is present in Fos303N843 and in the microviridin expression platform constructs pARW066-mdnABCDE-short and pARW071-mdnABCDE is given in A). The dots indicate, the fosmid carries additional genes from Nies843, totally about 43 kbp. B) shows the HPLC profiles of the microviridin test expression from the small microviridin expression platform vectors pARW066-mdnABCDE-short and pARW071-mdnABCDE compared to the fosmid Fos303N843. Microviridins were separated by RP-C18 chromatography and detected at 210 nm. The numbers indicate microviridin L (1) and its derivatives L1 (2), L2 (3), L3 (4).

MALDI-TOF MS, conducted by Dr. Keishi Ishida from the Leibniz Institute for Natural Product Research and Infection Biology, attested the presence of microviridin-like peptides, as shown in the following figure. Samples were measured in positive mode.

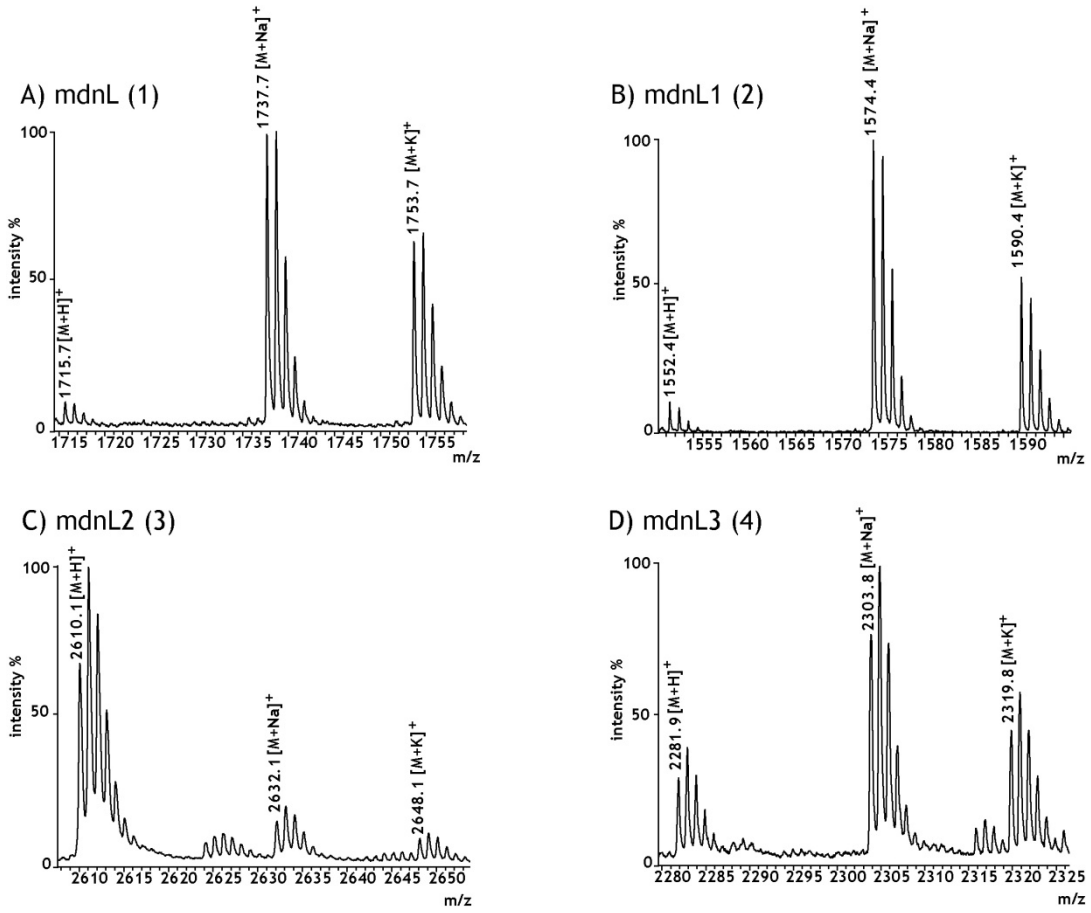

Figure 6-10: MALDI-TOF MS spectrum of microviridin L, L1, L2 and L3 expressed from the small microviridin expression platform pARW071-mdnABCDE

The presence of microviridin L is shown in A), L1 in B), L2 in C) and L3 in D) as m/z with ion charges $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$.

6.6 Analysis of the microviridin leader peptide

As a stable small expression platform was constructed, the investigation of the microviridin leader peptide could start. Till these days, not a single recognition sequence for the posttranslationally modifying enzymes or the protease has been reported from any microviridin leader peptide, which is why the discovery of those gained a legitimate interest. In order to analyze this aspect, sequences of the available microviridin precursor molecules were collected from the NCBI database and aligned using ClustalW, as shown in the following figure.

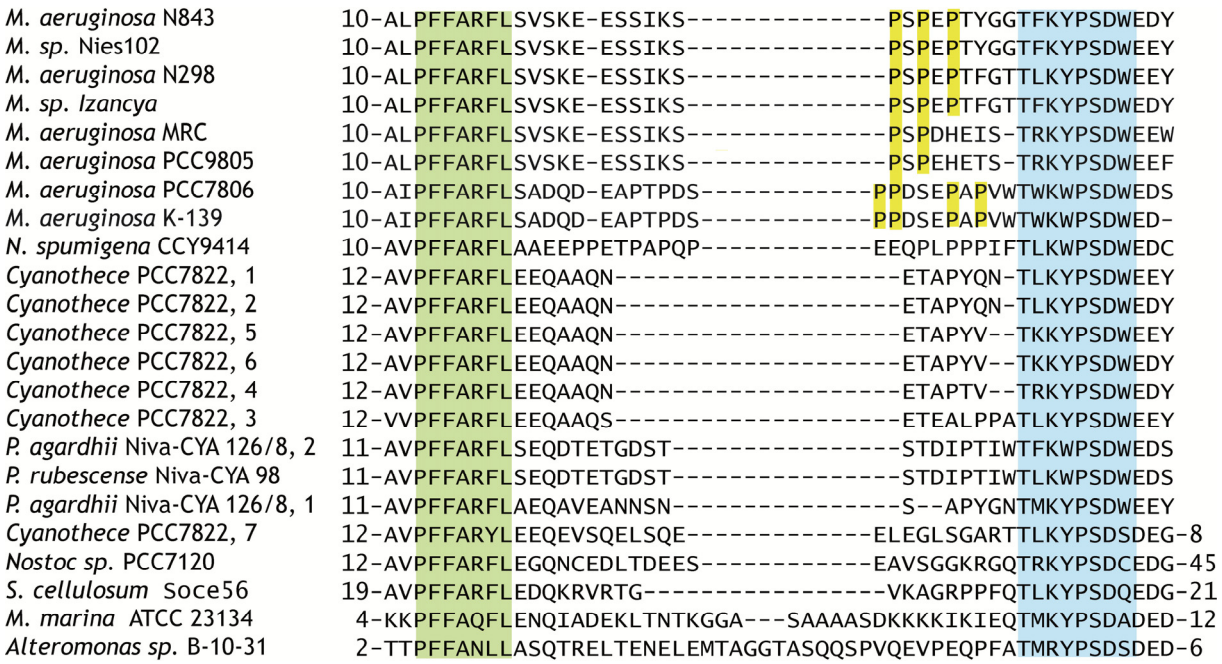

Figure 6-11: Microviridin precursor alignment

23 sequences collected from 16 potential and confirmed microviridin producers are shown in the alignment. Conserved PFFARFL and TxK(W/Y)PSDx regions, with x being different amino acids, are highlighted in green and blue boxes, whereas single yellow letters display a C-terminal proline-rich region in the leader peptides of *Microcystis* strains. Variable parts from the ends were replaced by numbers. M: *Microcystis*, N: *Nodularia*, P: *Planktothrix*, S: *Sorangium*, M: *Microscilla*

The alignment represented 3 interesting regions, first being the PFFARFL region, secondly the proline-rich region, both belonging to the leader peptide, and the TxK(W/Y)PSDx region (x being different amino acids), which is located in the microviridin core peptide.

6.6.1 N-terminal investigation of the microviridin leader peptide

The investigation of the N-terminal microviridin leader peptide started with the manipulation of single amino acids in the PFFARFL motif and C-terminal proline-rich region followed by additional techniques, as described in the next paragraphs.

6.6.1.1 Site-directed mutagenesis in the PFFARFL region of MdnA

By comparing all these precursor sequences it was noticed that *Microscilla marina* ATCC23134, a marine gliding bacterium and *Alteromonas sp.* B-10-31, also isolated from a marine habitat, carry aberrant amino acids in the PFFARFL region. As *Alteromonas sp.* B-10-31 is known to produce a microviridin-related compound called marinostatin that carries only the ω-ester bonds [Kanaori, et al., 2005], it was assumed, together with the fact that the PFFARFL motif is not ubiquitously present in ribosomally synthesized peptides that this region could serve as a recognition sequence for the ATP-grasp ligases. The genetic basis for marinostatin production furthermore does not encode an amide ligase.

Quik change mutagenesis (Agilent) was initiated using plasmid pARW071-mdnABCDE. First an arginine (R) was exchanged against an asparagine (N). HPLC analysis revealed a modified HPLC pattern (Figure 6-12). MALDI-TOF MS and MALDI-TOF/TOF MS (PSD) (PSD data not shown) were conducted by Dr. Keishi Ishida (HKI, Jena). Bicyclic microviridin L and L1, lacking the amide bond, was detected (Figure 6-13). With this result in hand, the remaining 6 amino acids in the PFFARFL region were successively exchanged, commonly using small and simple amino acids as glycine (G) and alanine (A). All recombinant strains were analyzed by HPLC (Figure 6-12) and mass spectrometry (MS data not shown). Independent from the amino acid exchanged, exclusively bicyclic microviridins namely L and L1, but also L2, L3 and incompletely processed versions, carrying the ω-ester bonds, appeared in even higher amounts compared to the original tricyclic microviridins (Figure 6-12 and Figure 6-14). Anyway microviridins carrying only the amide or one of each bond were not expected as the amide ligase is only active after the ester linkages are established [Philmus, et al., 2009]. Traces of tricyclic correctly processed microviridins were not noteworthy. In order to see what happens if several amino acids were exchanged, a double and a triple mutant of the PFFARFL region were constructed. HPLC was conducted followed by MALDI-TOF MS and MALDI-TOF/TOF MS (PSD) (MS data not shown). The resulting double mutant was still able to produce bicyclic microviridin L and L1 in matchable amounts, as seen before for the single mutants. The triple mutant showed a different result. Microviridin production was completely suppressed. Taken these data together, the PFFARFL region was shown to be important for both of the ATP-grasp ligases serving as a recognition sequence. MdnB is preferentially sensitive to changes in this region, which means the cooperation of MdnB with the leader peptide or between both of the ligases and the leader peptide is earlier disordered, whereas MdnC is more tolerant to mutations. At least 3 amino acids need to be exchanged to abolish the formation of the ester bonds.

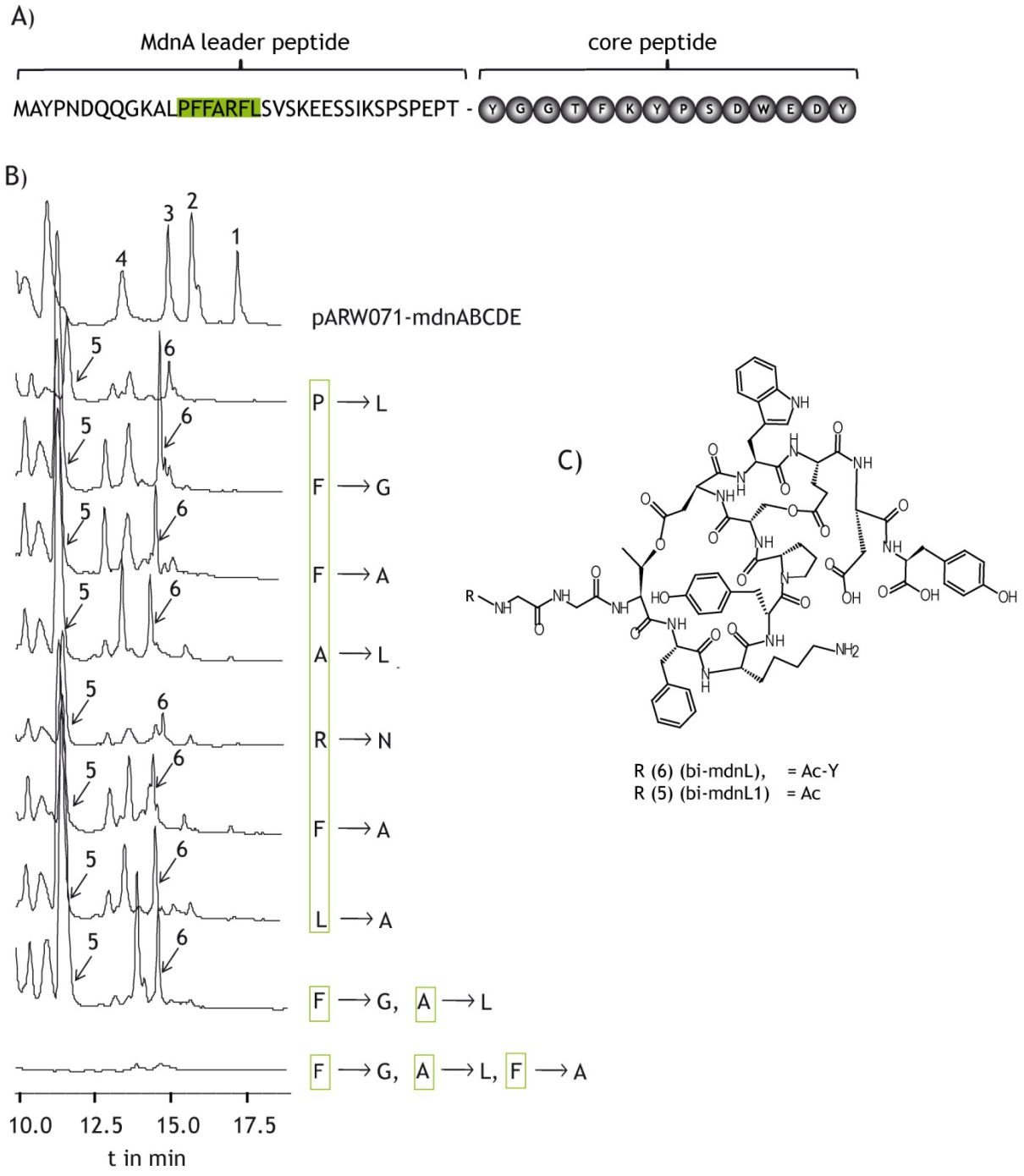

Figure 6-12: Microviridin production from PFFARFL motif mutants

The original PFFARFL sequence and its localization in the microviridin precursor molecule are given in A). An HPLC chromatogram of original pARW071-mdnABCDE and PFFARFL region mutants separated by RP-C18 chromatography, detected at 210 nm is shown in B). Amino acid exchanges are indicated adjacent to each of the HPLC runs. Produced microviridins are labeled with numbers 1-4 for tricyclic microviridin L, L1, L2, L3 and 5-6 for bicyclic microviridin L1 and L. The chemical structure of bicyclic mdnL (6) and L1 (5) produced from PFFARFL mutants is presented in C). Ac: acetyl

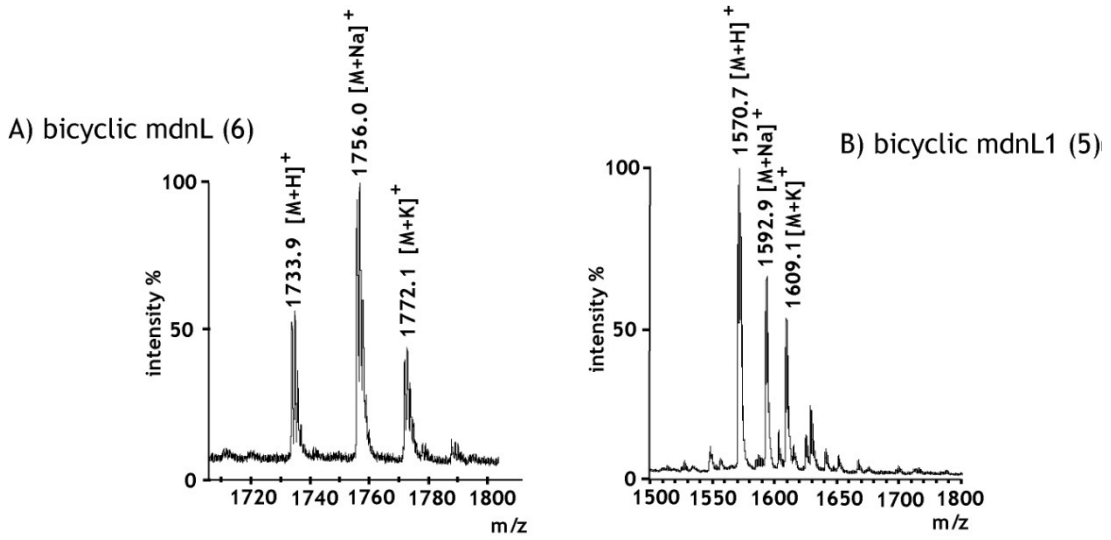

Figure 6-13: MALDI-TOF MS of bicyclic microviridins expressed from pARW071-mdnABCDE PFFARFL region mutants

The presence of bicyclic microviridin L (6) is shown in A) and bicyclic microviridin L1 (5) in B) as m/z with ion charges $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$

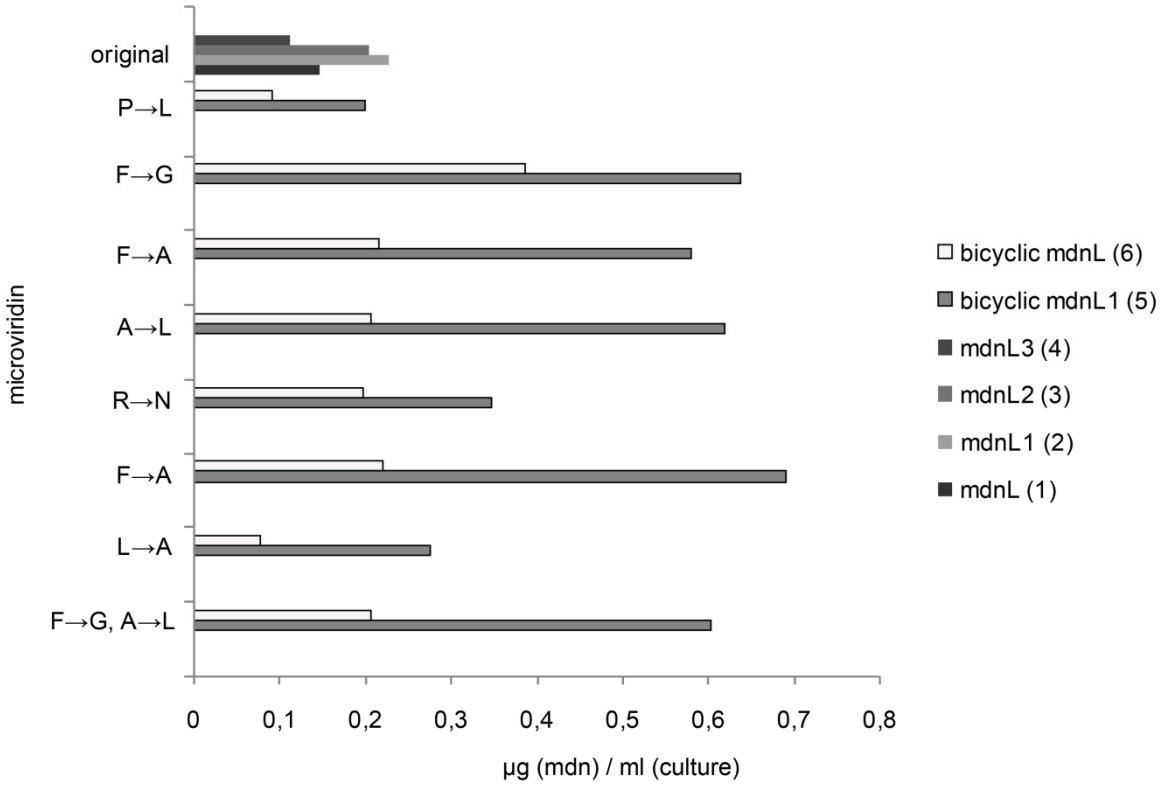

Figure 6-14: Quantification of tricyclic microviridin L, L1, L2, L3 (1-4) and bicyclic microviridin L1 and L (5-6)

Microviridins were produced from original pARW071-mdnABCDE and PFFARFL region mutants of pARW071-mdnABCDE. Values were determined by HPLC analysis including a standard of microviridin L, isolated from a recombinant source.

6.6.1.2 Studying peptide-protein interactions using SPR

To analyze interactions between the precursor molecule and the ATP-grasp ligases in more detail, surface plasmon resonance was chosen to unravel further functions of the PFFARFL region. Several attempts were made to express the microviridin precursor molecule of Nies298 in commonly used expression vectors like pET15b or from pETDuet-1-mdnA under native conditions, as already described (chapter 6.3), [Ziemert, et al., 2008]. Successful expression has never been seen, probably due to rapid degradation of the linear precursor peptide, which had not undergone cyclization through the ATP-grasp ligases. To circumvent the problem of proteolytic attack, peptide release into the periplasm was considered as a good strategy. The precursor molecule of Nies843 was expressed from pET40b(+), which is an expression vector using a DsbC-tag designed to create fusions to the enzymes that catalyze isomerization of disulfide bonds in the periplasm. Strong expression from that vector was observed, and although culture conditions were varied several times, degradation products were always present which is why pET40b(+) was also unsuitable for the purification of pure over-expressed MdnA under native conditions (data not shown). As the group of Hawaiian colleagues managed a faint but successful expression of the MdnA ortholog MvdE of *Planktothrix argardhii* from a pET-type vector [Philmus, et al., 2009], it was tried to express MvdE from a pET-vector. But unfortunately, these results were not reproducible, which led to the decision to use synthesized precursor molecules, named MvdE, MdnA(1)-original Nies843 and MdnA(2)—triple PFFARFL manipulated Nies843. Problems also appeared with the expression of MdnB under native conditions. No expression has been seen from pACYCDuet-1-mdnB from *Microcystis aeruginosa* Nies298, when cloned into the MCS I as a His-tag fusion protein, as described in paragraph 6.3. When expressed from MCS II in the same vector using an S-tag and enlarged culture volumes of 2 liters, relatively pure MdnB could be obtained in humble amounts (Figure 6-15, A). MdnC from *Microcystis aeruginosa* Nies298 was immediately and without any difficulty expressed from pACYCDuet-1-mdnC as an S-tag fusion protein under native conditions and in appropriate amounts (Figure 6-15, C). The presence of both proteins was verified by Western blot and immunodetection using S-protein HRP-conjugate (Figure 6-15, B and D). Immunodetection from total extracts gave very faint bands (data not shown).

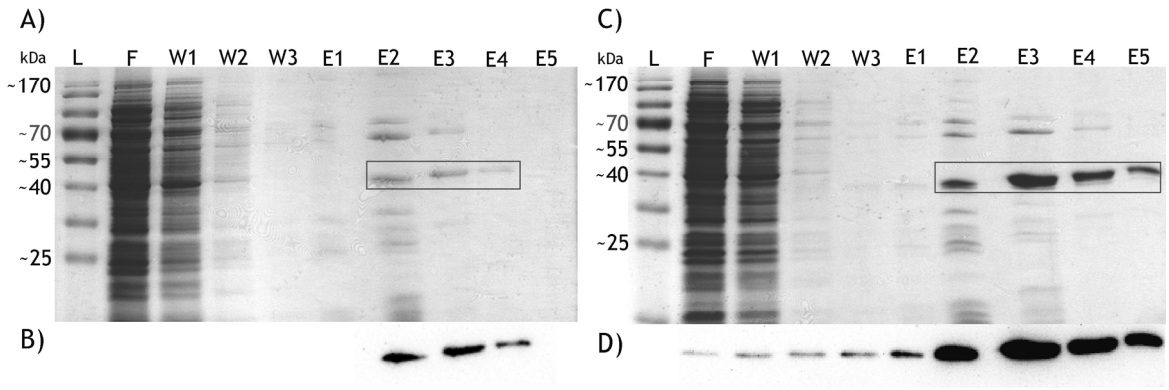

Figure 6-15: MdnB and MdnC purification and immunodetection against the S-tag

A) represents the SDS-PAG of Nies298 MdnB purification from pACYCDuet-1-mdnB using S-agarose. MdnB is shown in the black framed box. In B) the immunodetection of MdnB using S-protein HRP-conjugate is demonstrated. The SDS-PAG of Nies298 MdnC purification from pACYCDuet-1-mdnC using S-agarose is given in C). MdnC is shown in the black framed box. Immunodetection of MdnC using S-protein HRP-conjugate is shown in D). L: Page ruler prestained (Fermentas), F: flow-through, W1-W3: wash fractions 1-3; E1-E5: elutions 1-5

Once the proteins were purified and visualized by SDS-PAGE, fractions containing the most pure protein were pooled, concentrated and the buffer was changed to HEPES (pH 8.0) using Amicon filter units. Protein concentrations were measured by Bradford assay prior to the start of surface plasmon resonance analysis. The precursor molecules MvdE from *Planktothrix agardhii*, MdnA(1)-original Nies843 and MdnA(2)—triple PFFARFL manipulated Nies843 were immobilized on a CM5 sensor chip via amine coupling. MdnB and MdnC, injected in concentrations ranging from 0.15-20 µM, were analyzed for their capability to interact with the target. BSA was used as an initial control protein and later Mvn, ActM, TusA and POD were included. The following figure represents the obtained sensograms.

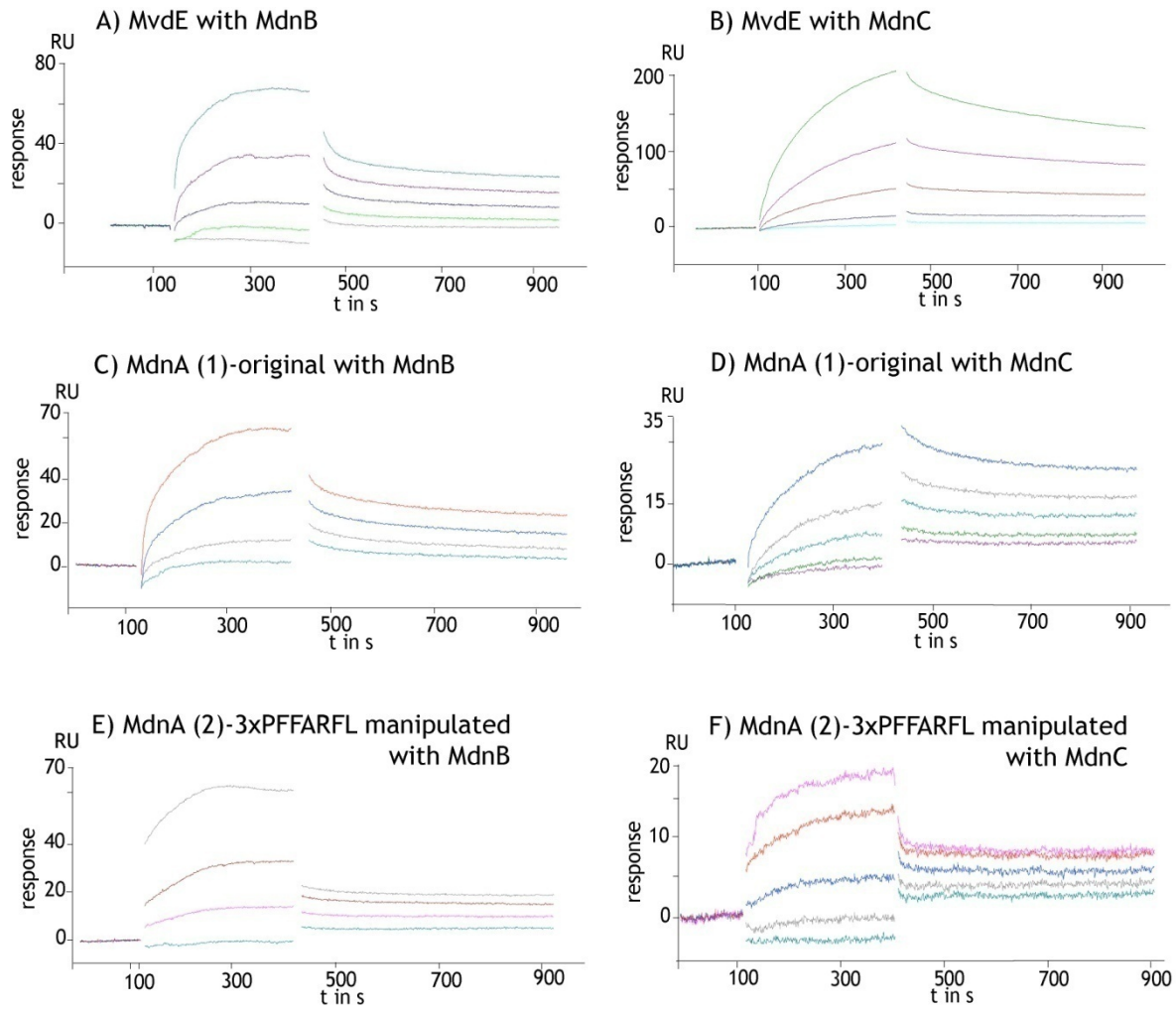

Figure 6-16: SPR analysis of specific protein interactions of MdnA variants with MdnB and MdnC

The microviridin prepeptides were immobilized on a CM5 sensor chip via amine coupling and tested for the interaction with MdnB and MdnC. Shown are MvdE with MdnB (A) and MdnC (B), MdnA(1)-original Nies843 precursor with MdnB (C) and MdnC (D) and MdnA(2)-triple-PFFARFl-manipulated precursor with MdnB (E) and MdnC (F). The proteins were introduced onto the surface at a concentration of 20.0, 10.0, 5.0, 2.5 and 1.25 µM. RU: response units.

Interactions between the ATP-grasp ligases and the 3 different microviridin precursor molecules were detected in each of the measurements, whereas no interaction with BSA was observed. In case of MvdE with MdnB and MvdE with MdnC (Figure 6-16, A/B) the response units were increased by increasing the concentration of MdnB and C, respectively, as shown through the different curve colors. The interactions of the original and manipulated MdnA from Nies843 with the ATP-grasp ligases showed a similar result, although it seems the most labile interactions with both ligases were observed with the manipulated precursor molecule. It was noticed that MdnC seems to make stronger interactions with the microviridin precursor molecules than MdnB. These data were in accordance to the results from the PFFARFL region mutagenesis and supported the hypothesis that the PFFARFL motif serves as a recognition sequence. Later in repetitive experiments further controls were included to rule out the possibility of nonspecific

interactions. The cyanobacterial proteins Mvn and ActM, as well as commercially available highly purified POD and the sulfurtransferase TusA from *E. coli* were used as control analytes. Unfortunately, some interactions were observed (data not shown). Nevertheless, differences were detected between the 3 tested microviridin prepeptides and the ligases, which make the results not completely invalueable, but they need to be verified with a different experimental technique, to ensure their relevance. PFFARFL region mutagenesis already showed the cooperation of the ATP-grasp ligases with the microviridin prepeptide (6.6.1.1).

6.6.1.3 Construction of a C-terminal leader peptide processing site

It has been seen from the fosmid and minimal expression system that microviridin production in *E. coli* always resulted in large amounts of incompletely processed products, compared to the correctly processed versions of microviridin L and L1. To improve the yield of correctly processed and to minimize the production of incompletely processed microviridins, first incubation conditions and media were varied several times without any success. Next, the construction of an engineered cleavage site was expected to solve the problem. Two enzymatic cleavage sites for trypsin (R$^{\vee}$YGG) and Factor Xa Protease (-IEGR$^{\vee}$IYGG-) and a chemical cleavage site for hydroxylamine (-N$^{\vee}$GG-) were successively constructed close to the proline region using site-directed mutagenesis, as illustrated in Figure 6-17. As shown in the HPLC profile, the insertion or the exchange of one amino acid decreased the amount of tricyclic microviridin L and L1, respectively, as shown for the construction of the trypsin and hydroxylamine cleavage sites. Tricyclic microviridin production completely disappeared when 3-5 amino acids were introduced for the Factor Xa processing site. Instead, low amounts of bicyclic microviridins were produced. Taking these facts together, it is obvious that the length of the leader peptide plays an important role in microviridin biosynthesis and cannot be simply changed. After the constructs were made, microviridins were purified from these candidates and incubated with the appropriate enzyme or chemical under various incubation conditions. However, under all tested conditions cleavage of the leader from the coding region could not be successfully shown. HPLC chromatograms remained unchanged before and after the digests were performed (data not shown). Probably, the cyclic structure of the microviridins and some steric arrangements effectively prevent cleavage. Microviridin itself is known to be a trypsin inhibitor which is why trypsin was used in excessive amounts. The lysine within the core was rather unexpected to be cleaved, as the microviridin cage-like structure was expected to prevent accessibility to this position.

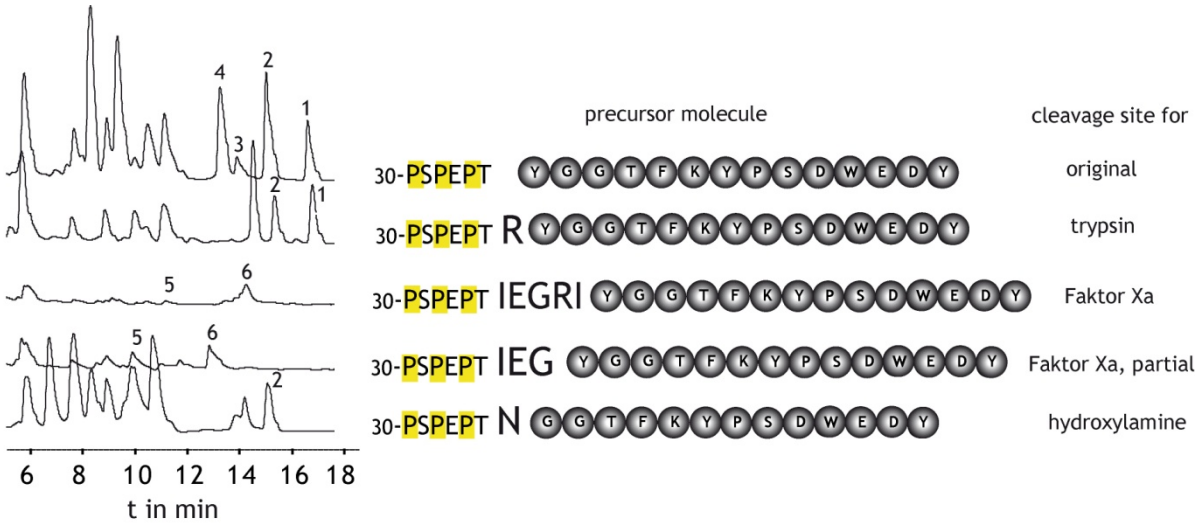

Figure 6-17: Mdn production in *E. coli* from MdnA carrying an engineered cleavage site

Microviridins were separated by RP-C18 chromatography and detected at 210 nm. The corresponding precursor with an amino acid insertion or an exchange, indicated in bold black letters, is shown next to the HPLC run. N-terminal regions of the leader peptide are omitted and given in numbers. Tricyclic microviridins L (1), L1 (2), L2 (3), L3 (4) are shown next to bicyclic microviridin L (6) and L1 (5.)

6.6.2 C-terminal investigation of the microviridin leader peptide

In *Microcystis* the C-terminus of the microviridin leader peptide is highly rich in prolines, as shown in yellow in Figure 6-11. From the literature several facts are known. First proline residues may serve as recognition sites for peptidases with specificity for proline-containing substrates [Vanhoof, et al., 1995]. Furthermore, the microviridin prepeptide exhibits some similar features commonly known from eukaryotic and prokaryotic signal peptides. There is a hydrophobic core region comprising 6-15 amino acid residues, which is essential for targeting and membrane insertion. The rather polar N-terminus has a positive net charge and is joined to the hydrophobic core. On the C-terminal side the hydrophobic core is flanked by a relatively polar region, which often contains helix-breaking residues as prolines or glycines [Martoglio and Dobberstein, 1998]. This information justified a closer look at the C-terminal region to analyze the importance for peptide cleavage and secondary structure formation in the microviridin biosynthesis.

6.6.2.1 Site-directed mutagenesis in the proline-rich region of *mdnA*

To analyze the impact of the 3 prolines at the C-terminus of the microviridin prepeptide, site-directed mutagenesis was again applied to construct a double and a triple proline region mutant. Prolines in the positions -2, -4, -6, starting from the core region, were exchanged against alanines. HPLC analysis showed that the double mutant was still able to produce correctly processed and cyclized microviridins, although simultaneously increasing amounts of bicyclic microviridin L (6) and L1 (5) were detected (Figure 6-18).

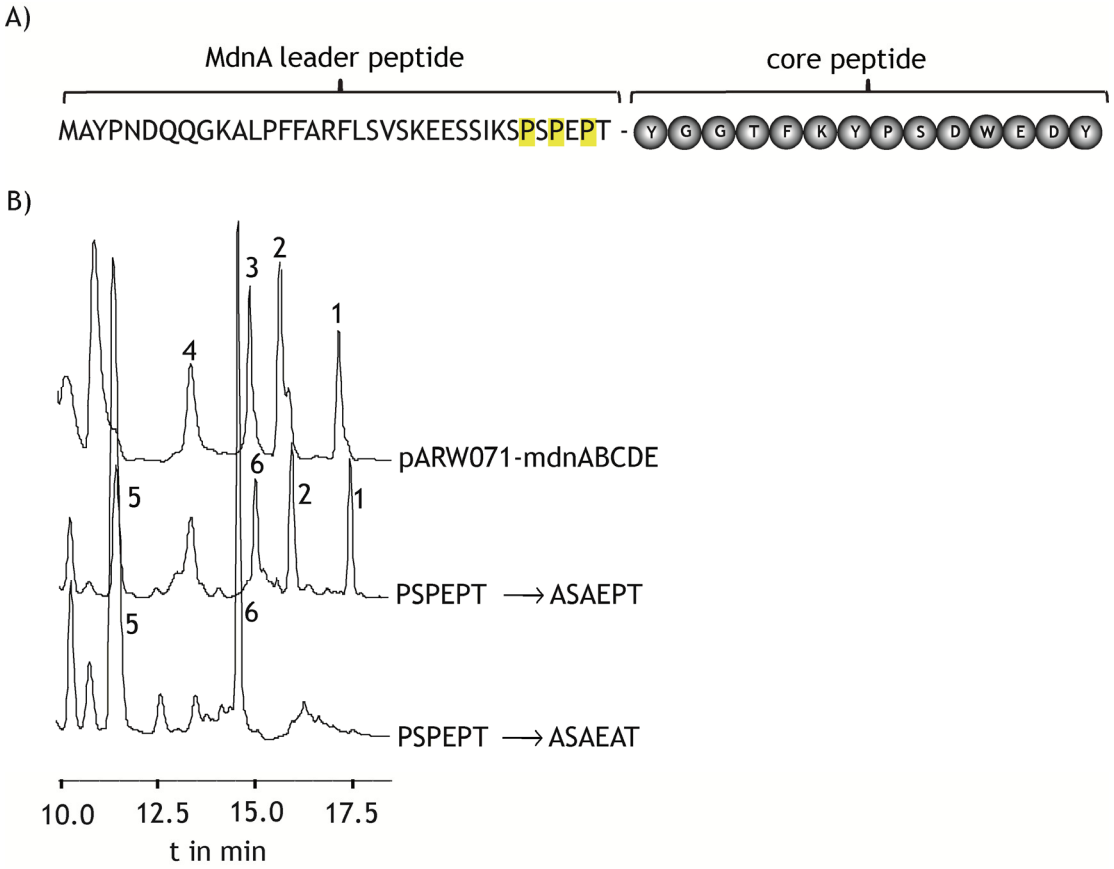

Figure 6-18: Microviridin production from proline region mutants

The proline-rich region at the C-terminus of the microviridin leader peptide is shown in yellow boxes in A). The HPLC profile of the leader peptide proline region mutants is shown in B). Microviridins were separated by RP-C18 chromatography and detected at 210 nm. The numbers 1-4 represent the tricyclic microviridins L, L1, L2 and L3. Number 5 and 6 indicate the bicyclic microviridins L1 and L. The amino acid exchanges are given on the right.

The triple proline region mutant lost the ability to produce tricyclic microviridins and instead produced large amounts of bicyclic microviridin L (6) and L1 (5). These data showed that the exchange of these amino acids had a minor influence on the cleavage efficiency and concision. It seems, a proline-specific endopeptidase is not primarily involved in the processing at that site. Nevertheless, the organisation of the C-terminal part of the leader peptide plays a key role in microviridin biosynthesis. It is assumed that the presence of repeated proline residues is possibly necessary for the formation of a ß-turn at the C-terminus of the leader peptide. Such a conformational arrangement might be required for the two PFFARFL-binding ATP-grasp ligases to establish the cyclizations.

6.7 Towards the understanding of the role of the ABC-transporter

As reported in this work and earlier [Ziemert, et al., 2008], the MRC-derived fosmid carrying only the genes *mdnABC*, was not able to produce correctly processed microviridin J, whereas Fos303N843 containing the complete cassette of *mdnABCDE* expressed completely processed microviridin L. The addition of extra copies of *mdnD* and *E* to

Fos303N843 resulted in increased microviridin production, as described in paragraph 6.4. Furthermore, complementation experiments with the MRC-derived fosmid and pCOLADuet-1-vector carrying *mdnDE* resulted in the production of microviridin J. These data suggested that effective microviridin production was somehow influenced and dependent on the presence of the ABC-transporter MdnE. These transporters are not only located in the same gene cluster as the structural gene but also exhibit dual functions. In bacteriocin biosynthesis they are involved in the removal of a double glycine containing leader peptide and the translocation across the cytosolic membrane [Havarstein, et al., 1995]. Thus, it was assumed that the ABC-transporter belonging to the microviridin gene cluster, although lacking a double glycine motif in the leader, is somehow involved in the maturation process of microviridins. Therefore, the role of MdnE was again analyzed using a small microviridin pDrive based platform. Analysis in the heterologous host was conducted, as no cyanobacterial mutant was available. PCR with primer pair P173-P97 was used to amplify the genes *mdnABCD* and about 714 bp of promoter region, as previously described for pARW071-mdnABCDE.

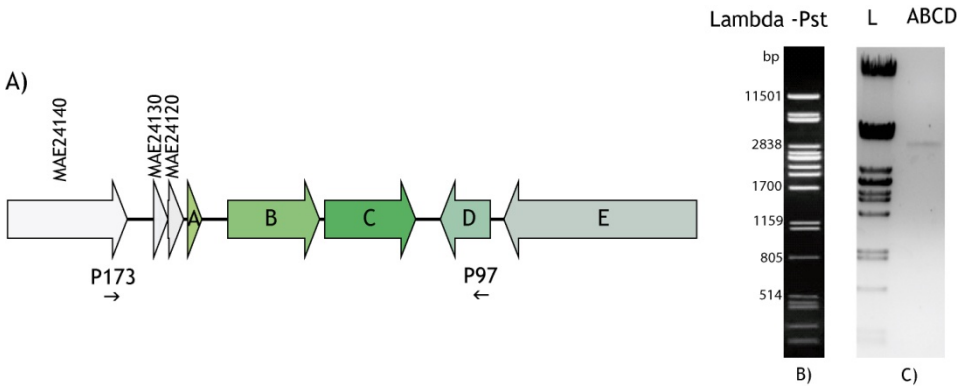

Figure 6-19: Agarose gels with the *mdnABCD* PCR product

A schematic representation of the amplified microviridin gene cluster is given in A). A Lambda-*Pst*I digest was used as a size standard, as shown in B) and L. The *mdnABCD* cassette was amplified with primer pair P173-P97 to include a long promoter region as given in C).

The gel-purified PCR product was cloned into pDrive (Qiagen) and several clones were sequenced. The obtained plasmid was named pARW081-mdnABCD (a plasmid maps is shown in the appendix). Microviridin production was analyzed by HPLC and mass spectrometry (MS data not shown), revealing a completely different expression pattern compared to pARW071-mdnABCDE. Microviridins were still produced but as a large proportion of bicyclic versions, which are incompletely processed at their N-termini. Thus, changes not only occurred in the cleaving but also in the cyclization process. Traces of bicyclic microviridin L1 are shown in peak number 5 in the following figure.

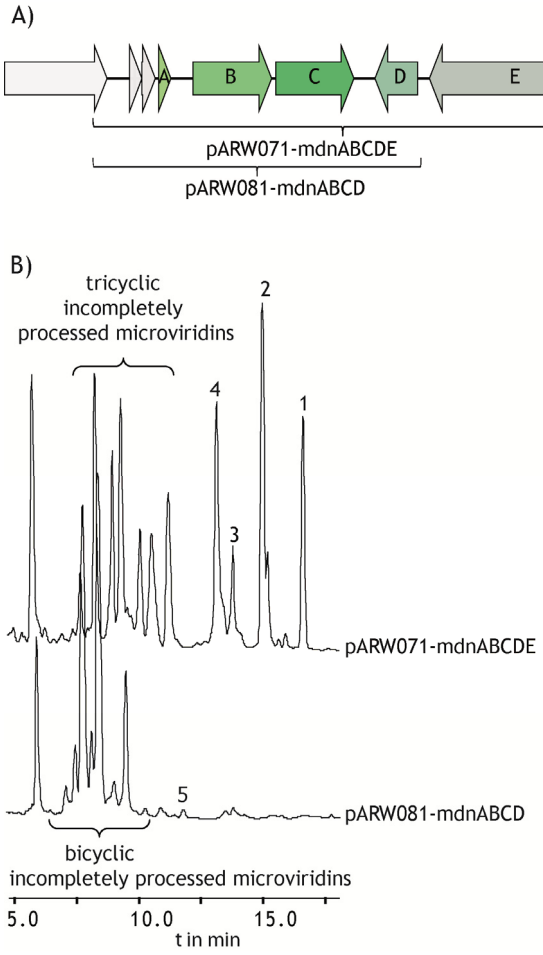

Figure 6-20: HPLC profile of microviridin expression from pARW081-mdnABCD

A schematic representation of pARW071-mdnABCDE and pARW081-mdnABCD constructs is shown in A). Microviridin production of pARW081-mdnABCD is shown next to pARW071 carrying the genes *mdnABCDE* in B). The numbers 1-4 represent the tricyclic microviridins L, L1, L2 and L3. Number 5 shows bicyclic microviridin L1. Microviridins were separated by RP-C18 chromatography and detected at 210 nm.

The phenomenon to produce only incompletely processed bicyclic microviridins was analyzed in 3 different strains of *E. coli* including the fosmid library host strain Epi300T1R as well as XL1 Blue and Top10 cells with always the same result. BLASTP showed MdnE is a membrane-spanning protein with a cytosolic ATP-binding domain. It seemed to be likely that MdnE assists in the formation of a putative microviridin biosynthesis and maturation complex associated to the membrane. MdnE may provide support for the correct positioning of the microviridin precursor to be cyclized by both or at least one ligase. Such membrane-associated complexes are known from ribosomally synthesized lantibiotics as nisin [Siegers, et al., 1996].

6.7.1 The role of MdnE in peptide processing and cleavage

To further investigate if MdnE-dependent peptide maturation and processing occurs during ATP-dependent transport, the sequence G-PSGR-GKS, popularly known as Walker A motif,

was manipulated. Therein, the essential lysine (K) was exchanged against an alanine (A), to prevent ATP hydrolysis, which is the driving force in active transport processes. ATP hydrolysis is a cooperative process between the two ATPase domains, meaning the activity of one domain activates the second domain. The knockout of one motif usually leads to a complete loss of transport function [Schneider, 2000]. Mutants generated by site-directed mutagenesis were still able to produce correctly processed tricyclic microviridins, indicating that peptide cleavage is not related to transport and does not require ATP hydrolysis, as shown in the following figure.

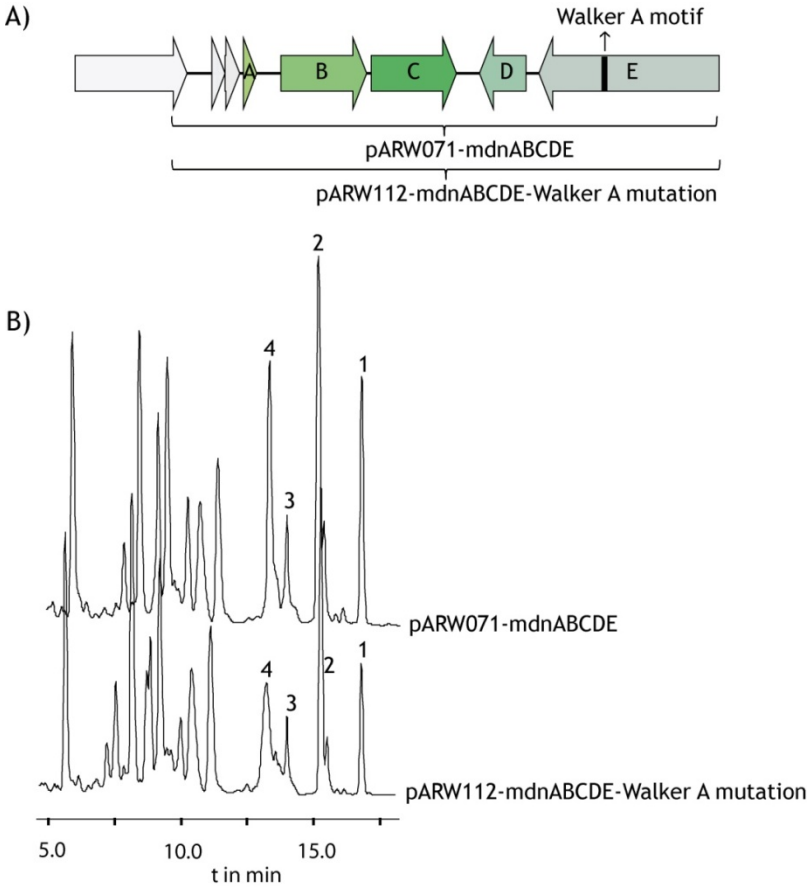

Figure 6-21: Microviridin expression from pARW112-mdnABCDE-Walker A motif mutant

A schematic representation of the two *E. coli* constructs carrying *mdnABCDE* genes with (pARW112) and without (pARW071) a Walker A motif mutation is given in A). Microviridin production of pARW112-mdnABCDE-Walker A mutation is shown next to pARW071 carrying the genes *mdnABCDE* in B). The numbers 1-4 represent the tricyclic microviridins L, L1, L2 and L3. Microviridins were separated by RP-C18 chromatography and detected at 210 nm.

6.7.2 The role of MdnE in microviridin transport

To analyze first if MdnE plays a role in microviridin transport independently from the processing process, the constructs pARW071-mdnABCDE and pARW081-mdnABCD were comparatively expressed in *E. coli*. The different cell compartments as the cytosol, the periplasm and the membrane fraction were analyzed by HPLC (Figure 6-22, A) and B)).

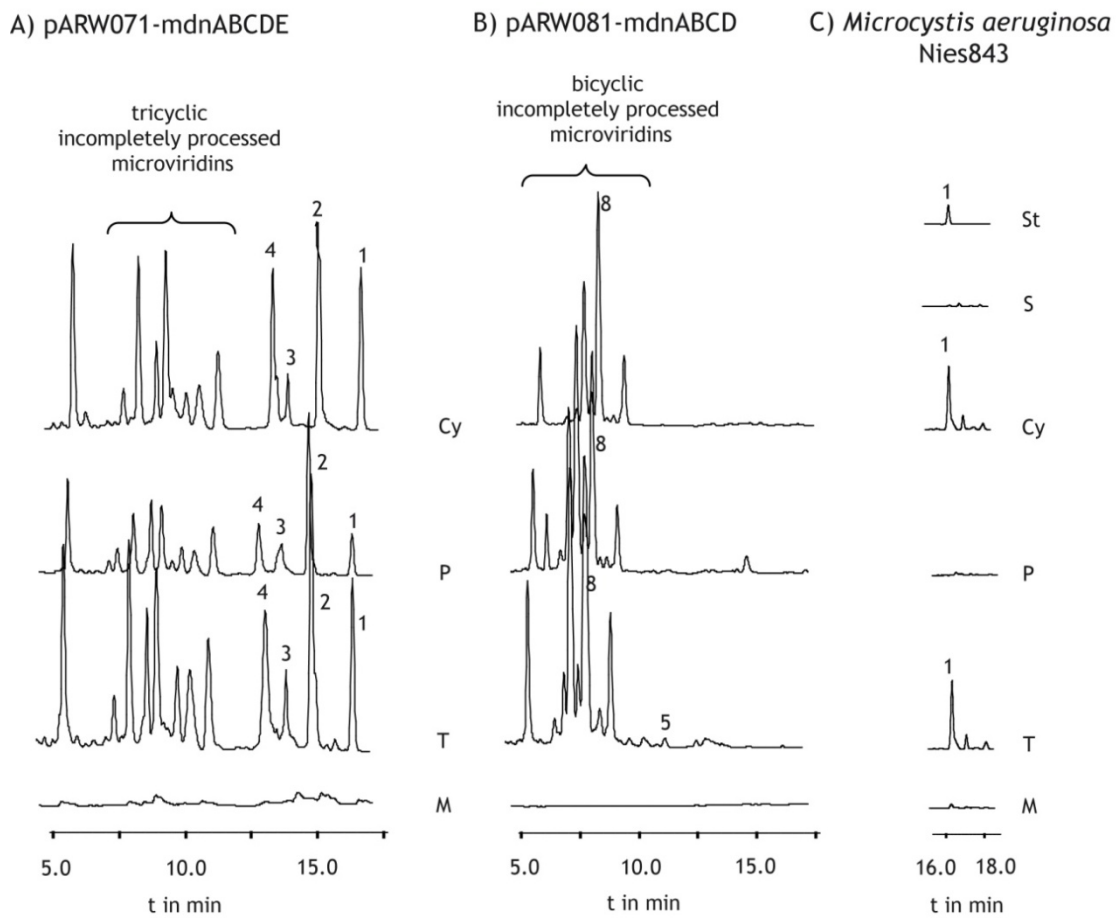

Figure 6-22: The role of MdnE in microviridin transport

An HPLC profile of the microviridin expression and detection in the cytoplasm (Cy), periplasm (P), membrane fraction (M) and the total extract (T) from *E. coli* construct pARW071-mdnABCDE is shown in A). An HPLC profile of the microviridin expression and detection in the cytoplasm (Cy), periplasm (P), membrane fraction (M) and the total extract (T) from *E. coli* construct pARW081-mdnABCD is given in B). The HPLC profile of the microviridin expression and detection in the culture supernatant (S), cytoplasm (Cy), periplasm (P), membrane fraction (M) and the total extract (T) from *Microcystis aeruginosa* Nies843 carrying the genes *mdnABCDE* compared to a microviridin L standard is represented in C). All microviridins were separated by RP-C18 chromatography and detected at 210 nm. Number 1-5 indicate tricyclic microviridin L (1), L1 (2), L2 (3), L3 (4) and bicyclic microviridin L1 (5) and L3 (8).

Microviridins were detected in the cytoplasm (Cy) and the periplasm (P) of *E. coli*, but not in the membrane fraction (M), independent from the presence of the ABC-transporter. In contrast, microviridins expressed from the original producer *Microcystis aeruginosa* Nies843, harboring the genes *mdnABCDE*, were only detected in the cytoplasm (Cy), as shown in Figure 6-22, part C. The same was observed when *Microcystis aeruginosa* Nies298 was analyzed (data not shown). SDS-PAGE was conducted to ensure that the periplasm and cytoplasm were strictly separated from each other, revealing a different pattern for both compartments in *E. coli* and cyanobacteria (data not shown). Transporter analysis in the cyanobacterium is so far impossible, because this strain is hardly amenable to genetic manipulations. Additionally the cyanobacterial culture supernatant (S) was analyzed but no microviridin-like peptides were detected under the applied standard growth conditions, as

shown in the figure above. Supernatant analysis of the *E. coli* cultures, using different minimal media with defined supplements, including M9 and a special minimal medium, failed under various growth conditions. Microviridins were detected in the cell pellet, when minimal medium was supplemented with LB medium, which made HPLC supernatant analysis impossible due to purification problems. In absence of LB as a supplement, *E. coli* cultures grew poorly and no microviridin-like peptides at all were detected after several days of culture incubation.

As similar amounts of microviridins were located in the periplasm from both *E. coli* strains (Figure 6-23) no matter if the transporter was present or not, it was assumed that microviridins may interact with another transport system. Microviridin amounts were calculated using triple biological replicates from HPLC purifications of microviridin L and L1 of pARW071-mdnABCDE, and bicyclic microviridin L3 from pARW081-mdnABCD.

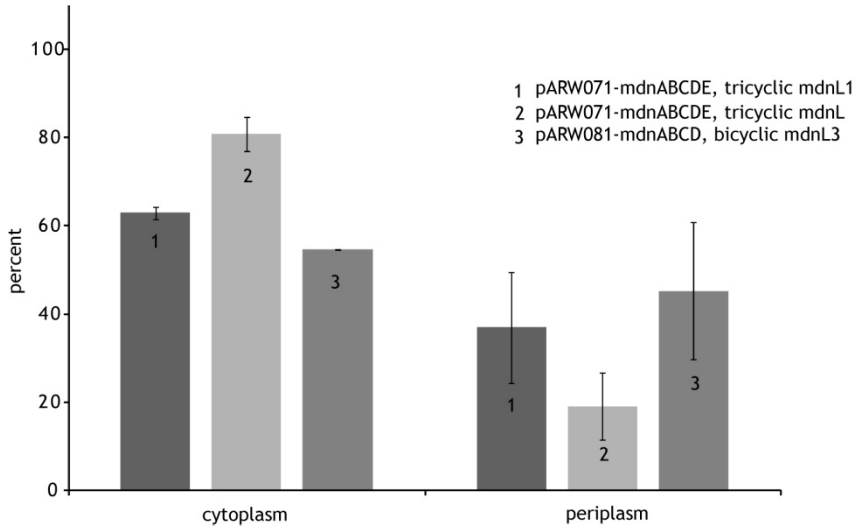

Figure 6-23: Microviridin quantification from periplasm and cytoplasm in *E. coli* constructs

Triple replicates of *E. coli* cultures carrying pARW071-mdnABCDE and pARW081-mdnABCD were analyzed by HPLC for their quantitative allocation of tricyclic microviridin L, L1 and bicyclic microviridin L3 in the cytoplasm and periplasm. Arrow bars indicate the standard deviation.

As another transport system for microviridins, the Sec system would be a nearby presumption. For that, biostatistic prediction tools as pSORTb and SignalP were employed to justify this assumption. The localization of MdnA and B was analyzed by pSORT but resulted in an insecure prediction. Both showed the possibility to be either located in the cytoplasm or the periplasm or even attached to the membrane. The use of SignalP, to predict potential signal sequences did not exclude the possibility of those in MdnA. Thus, the transport of MdnA could not be excluded. Taken together, so far under the conditions tried, there is no indication MdnE is neither involved in the transport of microviridins through the inner membrane in the cyanobacterium nor in the *E. coli* strains, but transport

to other parts of the cell or under different conditions is not entirely excluded. Transport might also occur under very special conditions not examined in the lab experiments.

6.7.3 The role of MdnE as a proposed scaffolding protein

To clarify the function of MdnE as a scaffolding protein, the presence and localization of the ATP-grasp ligase MdnB was analyzed in *E. coli* and the cyanobacterium. From experiments conducted by Katharina Makower (group of Prof. Dittmann, University of Potsdam) it was known that the expression levels of both microviridin cassettes in pDrive containing *mdnABCDE* and *mdnABCD* were akin to each other. *E. coli* constructs pARW071-mdnABCDE and pARW081-mdnABCD were analyzed using Western blot and immunodetection with the polyclonal antibody Anti-MdnB previously generated.

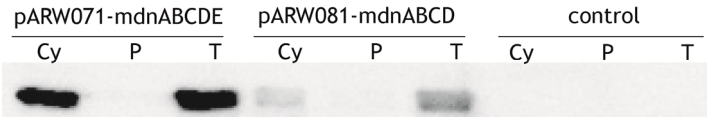

Figure 6-24: Subcellular localization and stability of MdnB in presence and absence of MdnE

pARW071-mdnABCDE and pARW081-mdnABCD were analyzed for the localization and stability of the ATP-grasp ligase MdnB in the cytoplasm (Cy), periplasm (P) and total extract (T) using Western blot analysis and immunodetection by the Anti-MdnB antibody. Plain *E. coli* cells were used as a control.

MdnB was mainly detected in the cytoplasm in both constructs, but obviously MdnB expression was significantly decreased in absence of the ABC-transporter MdnE. In cyanobacterial strains *Microcystis aeruginosa* Nies298 and Nies843 containing the complete microviridin expression cassette, the same result was obtained. Data were not shown as the bands are rather faint due to the lower copy number in the cyanobacteria compared to *E. coli*, where genes were expressed from a high copy plasmid. A cyanobacterial construct lacking the transporter is not available. The fact indicated one more time that MdnE plays an important role and may act as a scaffolding protein, stabilizing the putative microviridin biosynthesis complex or parts of it at the inner membrane on the cytosolic side.

6.7.4 *In vitro* activities of the ATP-grasp ligases and the ABC-transporter

The activity of the ATP-grasp ligases has been previously analyzed *in vitro* [Philmus, et al., 2008]. There, it has been shown that the two ligases, MvdD and MvdC from *Planktothrix agardhii* catalyze, although with rather low activity, the formation of a tricyclic compound from the 48-mer synthetic MvdE precursor peptide, with MvdD being the foremost working enzyme. Additionally, the acetyltransferase MvdB, has been shown to acetylate a 14-mer microviridin at the N-terminus, to result in the complete conversion into a product named

microviridin K. A similar approach was conducted by Nadine Ziemert [Ziemert, 2009] using the ATP-grasp ligases MdnB and MdnC and a synthesized MdnA precursor peptide from *Microcystis aeruginosa* Nies298, but unfortunately without any success of cyclization. From the current work several new insights into the mechanism of microviridin biosynthesis in *Microcystis* were gained and justified to repeat *in vitro* assays under modified conditions. MvdE, with its different organization in the C-terminal part of the leader peptide and the cytosolic domain of the ABC-transporter were included into a new serial of enzyme assays.

In order to establish an *in vitro* expression system, suitable for mechanistic studies and useful later for the investigation of certain mutations in the core peptide excluding the background of *E. coli,* an enzyme assay, similar to the one published by [Philmus, et al., 2008], was performed. As already described, there were certain difficulties to express and purify the microviridin precursor molecule heterologously. Thus, synthesized microviridin precursors (Genescript), MdnA(1)-original Nies843 precursor, MdnA(2)—triple PFFARFL manipulated precursor from Nies843, MdnA-original Nies298 precursor and MvdE from *Planktothrix* were analyzed together with the heterologously expressed ATP-grasp ligases from *Microcystis aeruginosa* Nies298 for cyclization events.

It has to be mentioned that all precursors but MdnA(1)-original were solubilized in either Tris-HCl or phosphate buffer pH 8.0. MdnA(1)-original was water insoluble and only the use of SDS in the enzyme assay mediated solubility. This concentration was assumed to be not inhibiting to the ligases, as concentration up to 0.1 % were used in previously reported reactions [Crimmins, et al., 2001]. Common detergents as Nonidet P-40 or Triton X did not improve solubility.

In none of these assays cross-linked microviridins were detected by HPLC and mass spectrometry. A mix of precursor from *Planktothrix* and ATP-grasp ligases from *Microcystis* did not result in any microviridin K-like compounds. Unconverted precursor molecules were detected without exception. *In vivo* at least the first cyclization in *Microcystis* occurs in the absence of MdnE, as demonstrated for microviridin expression from pARW081-mdnABCD. Nevertheless, as the ABC-transporter was shown before to be highly important for the correct assembly of microviridins, the cytosolic domain of the membrane protein was additionally expressed from a pACYC-Duet-1 vector and included in the enzyme assay. But again no cross-linked microviridin-like compounds were detected. As surface plasmon resonance experiments showed better interaction in the presence of higher ligase concentrations, different concentrations of ligases and the cytosolic domain of MdnE were tried. Unfortunately all these attempts showed no success.

6.8 Manipulation of the microviridin core peptide and bioactivity tests

The carboxy terminus of MdnA is representing the core sequence that is cleaved and enzyme-modified, resulting in the 14 amino acid short peptide microviridin, which exhibits bioactivity against serine proteases. In the past, Thomas Hemscheidt and coworkers already worked on the manipulation of certain amino acids in this area to analyze the substrate specificity of the ATP-grasp ligases *in vitro* [Philmus, et al., 2009]. They showed that the ligases exhibit a marginal flexibility for manipulations in the KYPSD region of MvdE. With the small microviridin expression platform pARW071-mdnABCDE it was possible to further manipulate and analyze the core region *in vivo* to broaden and deepen the knowledge about the substrate specificity of the ligases. The possibility to cut and paste precursor peptides of cryptic microviridins into the established expression system was analyzed. Furthermore, the bioactivities of the resulting mutants were dissected to determine certain amino acid positions to be highly important to maintain, to increase or even change bioactivities through the introduction of new and prospectively unusual residues. This information could guide the construction of microviridin peptide libraries with potential pharmacological use in the future.

6.8.1 The impact of crosslink reduction for production yield and bioactivity

First of all, the bioactivities of tricyclic heterologously expressed microviridins L, L1, L2 and L3 were analyzed against eukaryotic serine proteases as trypsin, chymotrypsin and elastase by Dr. Keishi Ishida (HKI, Jena). Cultures of about 2 liters of *E. coli* carrying the fosmid Fos303N843 microviridin expression platform were grown for several hours. In comparison, expression of sufficient amounts from the original producer would take weeks. Microviridins were purified and bioactivity tests were conducted. It has been shown that microviridin L and its derivatives exhibit moderate bioactivity in the micromolar range against serine proteases, compared to other microviridins reported in the literature [Ziemert, et al., 2010]. Stable amounts of bicyclic microviridins lacking the amide bond were successfully produced from the PFFARFL region single and double mutants, described in paragraph 6.6.1.1. To determine the impact of the lactam ring on the bioactivity, these candidates were tested against the same proteases, but additionally subtilisin was included. Indeed, bicyclic microviridin mutants were slightly more potent against subtilisin, whereas the other inhibitory characteristics compared to tricyclic microviridins were almost unchanged. Thus, the influence of the lactam ring forming amide bond on the inhibitory pattern of microviridins has been shown to be marginal. To further analyze the impact of the number of cyclizations on stability and bioactivity, a mutant producing monocyclic microviridins has been constructed by site-directed mutagenesis. A serine residue (S) of the KYPSD region was exchanged against an alanine (A). Similar attempts

were previously reported by [Philmus, et al., 2009]. Several mutants were analyzed by HPLC and mass spectrometry (MS data not shown). They showed almost no production of monocyclic microviridin L1 type.

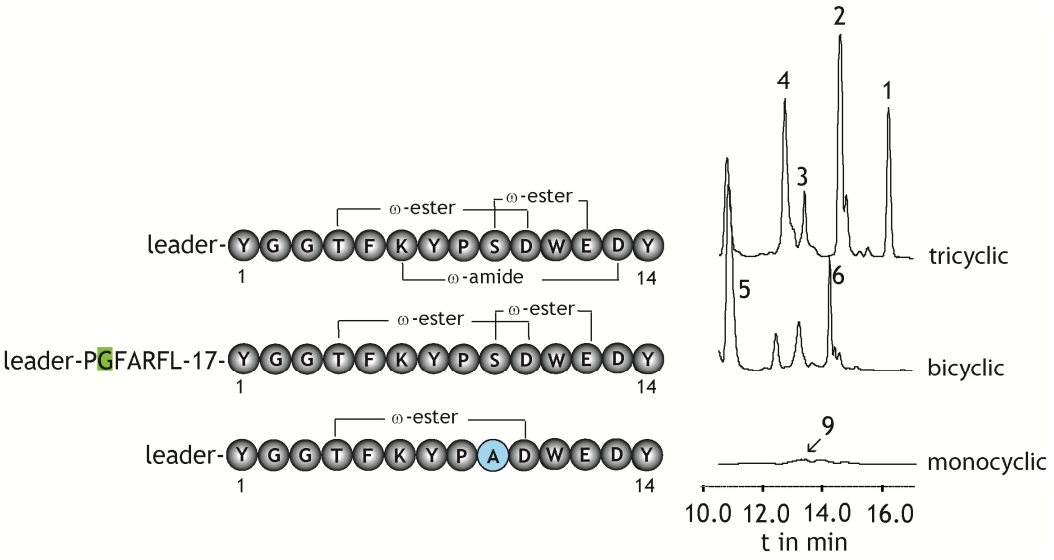

Figure 6-25: Alterations in the cage-like structure of microviridins

Tricyclic, bicyclic and monocyclic microviridins were produced from precursor molecules shown on the left. Exchanged amino acid residues are indicated in green and blue. The amino acid sequence of the leader was omitted, unless the mutation was located there, as shown in the middle. The PGFARFL mutation is shown as one example for bicyclic microviridins. Numbers in the leader sequence explain the number of the following amino acids belonging to the leader. The HPLC profile corresponding to the precursor sequences is shown on the right. Numbers indicate tricyclic microviridin L (1), L1 (2), L2 (3), L3 (4), bicyclic microviridin L (6) and L1 (5) and monocyclic microviridin L1 type (9). All microviridins were separated by RP-C18 chromatography and detected at 210 nm.

Again large *E. coli* mutant cultures were grown for 10 hours. Microviridins were purified and analyzed for their inhibitory potential. N- and C-terminal degraded structures were mainly detected, indicating that monocyclic microviridins, lacking the smaller ester and the amide bond, are more unstable. Nevertheless, bioactivity against chymotrypsin has been faintly increased, whereas no inhibitory effect against subtilisin, trypsin and elastase was discovered. Thus, the formation of the smaller lacton ring is of high importance to maintain structure stability and bioactivity.

Table 6-1: Serine protease inhibitory activity of tri-, bi- and monocyclic microviridins

		inhibitory activity (IC_{50}) in µmol/l			
sequence	cyclization	elastase	chymotrypsin	trypsin	subtilisin
Ac-YGGTFKYPSDWEDY	tri-mdnL	>58	42	58	8.7
Ac-GGTFKYPSDWEDY	tri-mdnL1	>64	24	>64	5.8
Ac-YGGTFKYPSDWEDY	bi-mdnL	>57	>57	>57	2.3
Ac-GGTFKYPSDWEDY	bi-mdnL1	>63	41	>63	2.5
GTFKYPADWE-OH	mono-mdn	>63	16	>63	>63
Ac-GGTFKYPADWE-OH	mono-mdn	>84	20	>84	>84

6.8.2 Mutations in the TxKxPSDx region

In order to analyze how far changes in the highly conserved TxKxPSDx region can be expanded and how bioactive the resulting mutants are, this region has been manipulated step by step using site-directed mutagenesis in the small microviridin expression platform pARW071-mdnABCDE. First, aromatic phenylalanine in position 5 (F5) in the core region has been exchanged against hydrophobic leucine (L5). The most potent microviridins, in terms of bioactivity, carry a leucine at this position, whereas microviridin L originally has a phenylalanine (F). Indeed, the resulting mutant produced stable amounts of tricyclic microviridin L and L1 corresponding to the amino acid exchanges.

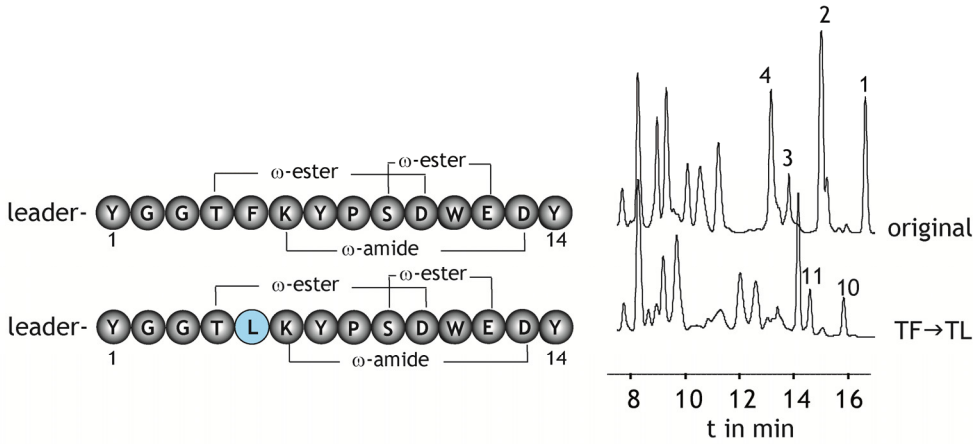

Figure 6-26: Microviridin production from TF→TL mutant

Original and TF→TL mutated microviridins were produced from precursor molecules shown on the left. The exchanged amino acid residue is indicated in blue. The HPLC profile corresponding to the precursor sequences is shown on the right. Numbers indicate tricyclic microviridin L (1), L1 (2), L2 (3), L3 (4) and microviridin L TF→TL type (10) and microviridin L1 TF→TL type (11). All microviridins were separated by RP-C18 chromatography and detected at 210 nm.

The bioactivity was increased about a 100-fold against elastases and to a lower degree also against chymotrypsin and subtilisin. The activity against trypsin remained unchanged.

Thus, a strong inhibitory activity was obtained by intentionally altering the phenylalanine in position 5 by introducing a leucine.

Table 6-2: Serine protease inhibitory activity of TF→TL microviridin mutant

		inhibitory activity (IC_{50}) in µmol/l			
sequence	cyclization	elastase	chymotrypsin	trypsin	subtilisin
Ac-YGGTFKYPSDWEDY	tri-mdnL	>58	42	58	8.7
Ac-GGTFKYPSDWEDY	tri-mdnL1	>64	24	>64	5.8
Ac-YGGTLKYPSDWEDY	tri-mdnL type	0.433	17	>63	3.4
Ac-GGTLKYPSDWEDY	tri-mdnL1 type	0.133	18	>59	3.8

In order to further analyze the possibility to mutate the core region, rarely occurring amino acids detected in the natural library of sequences from the KxPSDx region, as shown in the alignment below, where chosen to be introduced into the precursor sequence of microviridin L. The microviridins from the organisms harboring the presented precursor sequences are so far cryptic.

Figure 6-27: Precursor sequences of cryptic microviridins carrying rare amino acids

Microviridin encoding precursor sequences and their hosts are shown. Rarely occurring aberrant amino acids in the KxPSDx region are highlighted in blue. The amino acid sequences were obtained from the NCBI Blast server and aligned using ClustalW. M.: *Microcystis, sp.: species*

The small microviridin platform pARW071-mdnABCDE was again used to alter the highly conserved KYPSDx core sequence of MdnA by a genetic engineering technique. The aromatic tyrosine in position 7 (Y7) was exchanged against aromatic residues like tryptophane (W7) and phenylalanine (F7), whereas original aromatic tryptophane in position 11 (W11) was replaced by hydrophilic cysteine (C11) or serine (S11). The resulting mutants were analyzed by HPLC, as shown in the following figure and verified by mass spectrometry (data not shown).

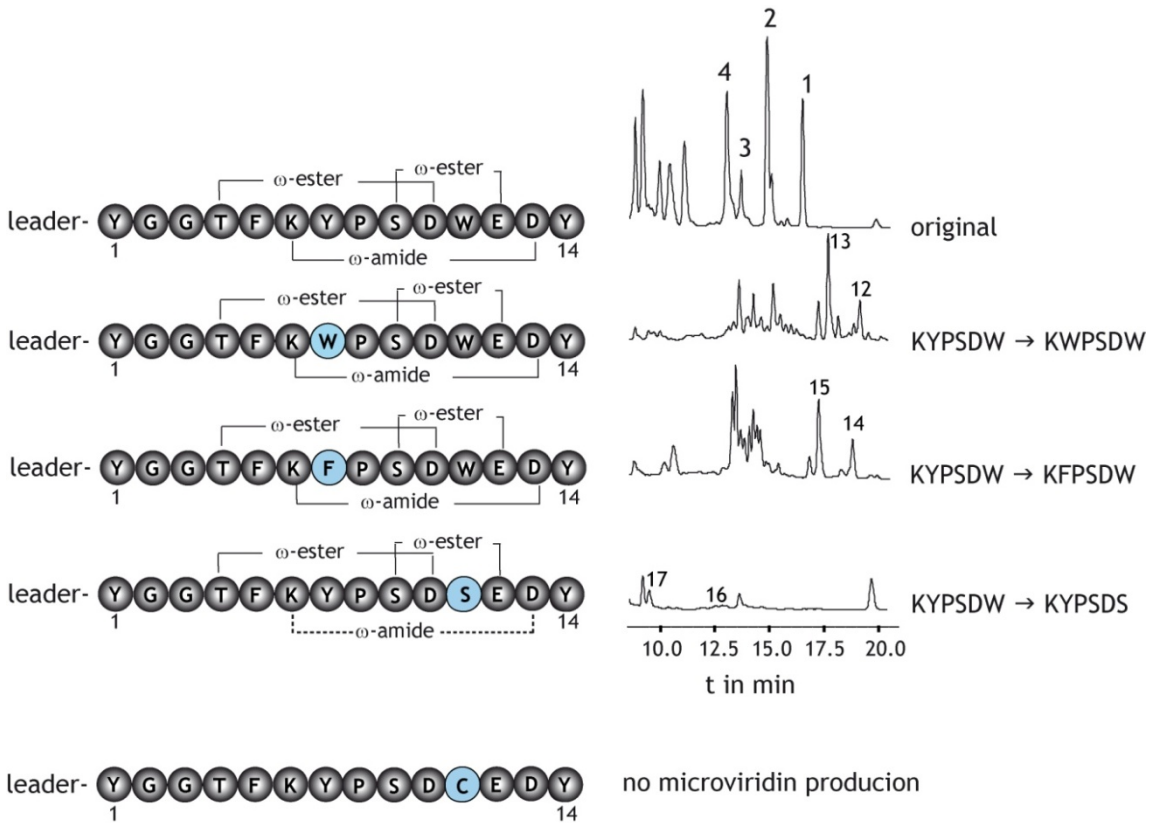

Figure 6-28: Manipulations in the KxPSDx region of microviridin L

Original and KxPSDx mutated microviridins were produced from precursor molecules shown on the left. Exchanged amino acid residues are indicated in blue. The HPLC profile corresponding to the precursor sequences is shown on the right. Numbers indicate tricyclic microviridin L, L1, L2, L3 (1, 2, 3, 4), microviridin L and L1 KWPSDW type (12, 13), microviridin L and L1 KFPSDW type (14, 15) and traces of microviridin L and L1 KYPSDS type (16, 17). KYPSDC mutant did not produce any microviridin-like compounds. All microviridins were separated by RP-C18 chromatography and detected at 210 nm.

New tricyclic microviridin L and L1 types corresponding to the amino acid exchanges in position 7 were stably produced from KWPSDx and KFPSDx mutants. Thus, ligase flexibility has been shown for position 7 when amino acids with similar properties compared to the original were introduced into the peptide chain. In contrast, substitutions in position 11 led to a breakdown of microviridin biosynthesis. Trace amounts of bicyclic (not shown) and tricyclic mdnL and L1 structures were mainly detected by mass spectrometry (data not shown) from the KYPSDS mutant. For the insertion of cysteine, carrying a highly reactive thiol group in the side chain, no microviridin-like peptides were detected. Thus, the amino acid exchanges in position 11, using amino acids with more distant characteristics, showed a stronger effect. Due to the low microviridin production level from KYPSDS mutant, studies into the inhibitory profile were only conducted for KWPSDx and KFPSDx mutants.

Table 6-3: Serine protease inhibitory activity of KWPSD and KFPSD mutants

		inhibitory activity (IC_{50}) in µmol/l			
sequence	cyclization	elastase	chymotrypsin	trypsin	subtilisin
Ac-YGGTFKYPSDWEDY	tri-mdnL	>58	42	58	8.7
Ac-GGTFKYPSDWEDY	tri-mdnL1	>64	24	>64	5.8
Ac-YGGTFKWPSDWEDY	tri-mdnL type	>58	>58	>58	>29
Ac-GGTFKWSDWEDY	tri-mdnL1 type	>64	20	>64	5.0
Ac-YGGTFKFPSDWEDY	tri-mdnL type	>59	24	>59	6.6
Ac-GGTFKFPSDWEDY	tri-mdnL1 type	>65	13	>65	5.1

As shown by the results in Table 6-3 compared to original microviridin L, there were no remarkable alterations detected in the inhibitory profile of the KWPSD and KFPSD mutants against the tested serine proteases. Thus, position 7 seemed to be not primarily important for the bioactivity.

6.8.3 Construction of a core peptide detected from a field sample

Previously, a microviridin precursor encoded in the amino acid sequence YNVTLKYPSDWEEF was detected from field sampling, occurring in more than half of 50 analyzed clones, from the Braakman reservoir (Netherlands) and from the Baltic Sea in Mecklenburg-Vorpommern (Germany) [Ziemert, 2009]. It was assumed that the high abundance of this particular amino acid sequence might correlate with beneficial properties in terms of bioactivity for the harboring organisms. Several rounds of site-directed mutagenesis were performed to convert the small microviridin expression platform pARW071-mdnABCDE, carrying the precursor sequence YGGTFKYPSDWEDY of *Microcystis aeruginosa* Nies843 into the new sequence. The final construct as well as a construct carrying the sequence YNVTLKYPSDWEEY was analyzed by HPLC and mass spectrometry (MS data not shown). Tricyclic microviridins from both engineered precursors were produced in *E. coli* cells, although in very little amounts compared to the original, as shown in the following HPLC profile.

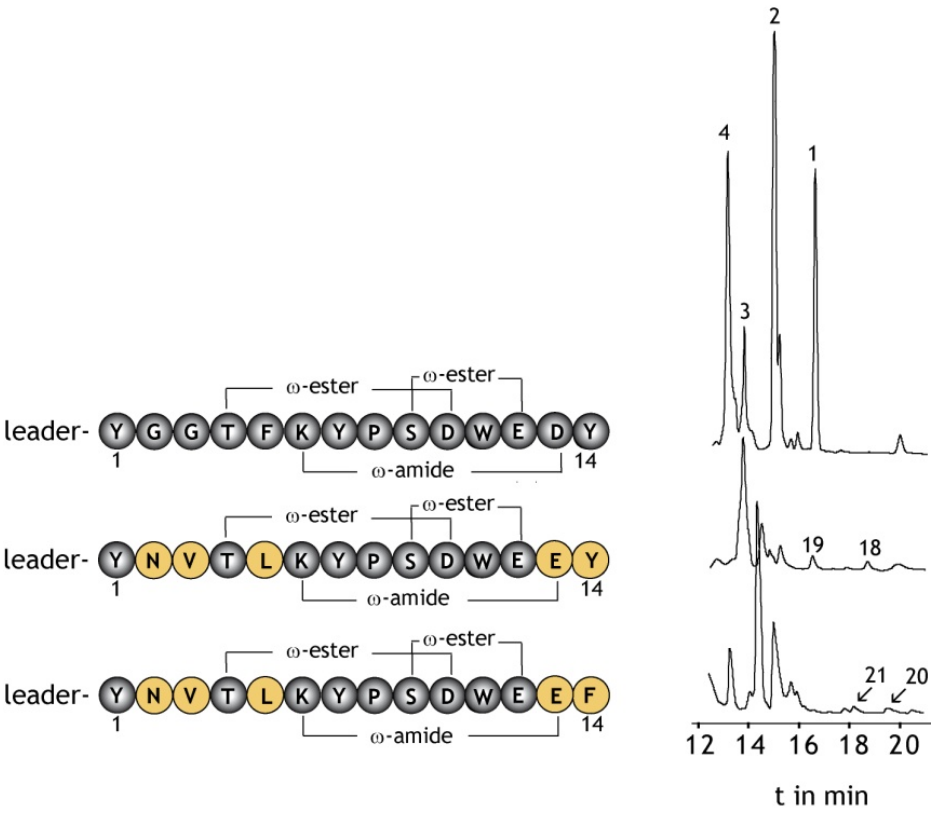

Figure 6-29: Microviridin production from an unknown core peptide detected in the field

Original and microviridin L/L1 field Y14/F14 type microviridins were produced from precursor molecules shown on the left. Exchanged amino acid residues are indicated in orange. The HPLC profile corresponding to the precursor sequences is shown on the right. Numbers show tricyclic microviridins, microviridin L field Y type (18), microviridin L1 field Y type (19), microviridin L field F type (20) and microviridin L1 field F type (21). All microviridins were separated by RP-C18 chromatography and detected at 210 nm.

Large 6 liter *E. coli* cultures carrying both field sample core peptides were grown and microviridins were isolated to determine their inhibitory profile. It was noticed that microviridins from large cultures were degraded at the N-terminus and did undergo partial methylation as shown with * in the table below. Both has never been detected from smaller cultures. Degradation was supposed to result from technical cooling problems during the purification process, as microviridins are reported to react sensitively to higher temperatures [Rohrlack, et al., 2003]. Indeed, especially the inhibitory activity towards elastase was significantly increased from the micromolar to the nanomolar range. Thus, the initial hypothesis seems to be very likely. The predominant microviridin variant was shown to be the very active. The stepwise mutagenesis showed that the amino acid in position 5 is highly important for the bioactivity. The other bioactivities remained unchanged or were even decreased.

Table 6-4: Serine protease inhibitory activity of YNVTLKYPSDWEE(Y/F) mutants

		inhibitory activity (IC_{50}) in µmol/l			
sequence	cyclization	elastase	chymotrypsin	trypsin	subtilisin
Ac-YGGTFKYPSDWEDY	tri-mdnL	>58	42	58	8.7
Ac-GGTFKYPSDWEDY	tri-mdnL1	>64	24	>64	5.8
VTLKYPSDWE*EY	tri-mdn	4.6	>63	>63	>63
Ac-NVTLKYPSDWE*EY	tri-mdn	0.379	>60	>60	13
VTLKYPSDWEEF	tri-mdn	4.8	37	>69	>69
VTLKYPSDWEEF	bi-mdn	5.0	68	>68	>68

* indicates the formation of a methylester

6.8.4 Cut and paste of microviridin precursor molecules

In order to analyze the possibility to express cryptic microviridin core sequences with their own corresponding leader peptides in the backbone of pARW071-mdnABCDE, the small microviridin expression platform was again manipulated by site-directed mutagenesis. Performing several rounds of mutagenesis, *Ehe*I and *Aat*II restriction sites were introduced into the intergenic space surrounding the 5´and 3´ends of *mdnA*. The construct was named pARW089-mdnA-exchange platform. Microviridin L production was verified by HPLC. It was noticed from all candidates analyzed, that production levels were faintly decreased compared to the pARW071-mdnABCDE platform. Cryptic microviridin precursor molecules of *Nostoc sp*. PCC7120 (GenBank: BAB78097.1), *Cyanothece sp*. PCC7822 (NCBI Reference Sequence: YP_003890018.1 and YP_003890026.1) and *Microcystis aeruginosa* PCC7806 (GenBank: CAO86268.1) were amplified by PCR using primers with the appropriate restriction site at the 5´-end. Products were first cloned into pDrive and transferred from there into the microviridin expression platform, resulting in the plasmids named pARW097-PCC7120-1, pARW098-PCC7822-2, pARW099-PCC7822-3 and pARW100-PCC7806-4. Candidates were analyzed for their ability to express the obtained precursors, as shown in Figure 6-30. No microviridin production was detected from the structurally most distant precursor of *Nostoc sp*. PCC7120 carrying a double glycine motif and three potential microviridin core sequences. Traces of bicyclic, acetylated microviridins of the mdnL and L1 type (Ac-YQNTLKYPSDWEDY, Ac-QNTLKYPSDWEDY) were detected from *Cyanothece sp*. PCC7822-2 precursor. Traces of nonacetylated, bicyclic N-terminally shortened microviridins (PIFTLKFPSDWEDS) were produced by *Cyanothece sp*. PCC7822-3 precursor. The presence was mainly detected by mass spectrometry (data not shown) as peaks are hardly visible in the HPLC chromatograms. Bicylic, N-terminally truncated microviridins (WTWKWPSDWEDS) were detected from the most closely related *Microcystis*

aeruginosa PCC7806 prepeptide. Tricyclic microviridins were never detected from any of the precursors.

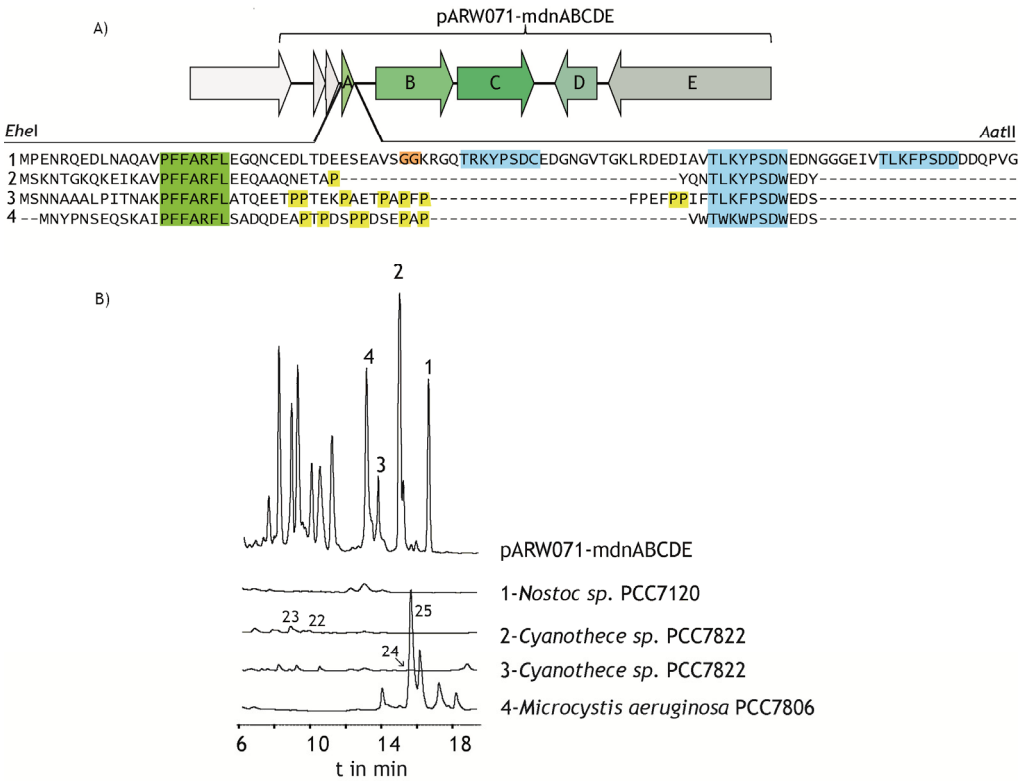

Figure 6-30: Expression of cryptic mdn precursors using the pARW071-mdnABCDE platform

A) MdnA from Nies843 was excised from the pARW089-mdn-exchange platform using *Ehe*I and *Aat*II and replaced by cryptic microviridin precursors from *Nostoc sp.* PCC7120-1, *Cyanothece sp.* PCC7822-2/3 and *Microcystis aeruginosa* PCC7806-4. Cryptic precursors are shown as protein sequences. The conserved PFFARFL region is shown in green, helix-breaking polines are shown in yellow, a double GG-motif is shown in orange and the TxKxPSDx region is shown in blue. B) HPLC profile of microviridin production from the inserted precursor molecules. No mdn production was detected from PCC7120-1. Traces of bicyclic microviridins, microviridin L PCC7822-2 type (22), microviridin L1 PCC7822-2 type (23), microviridin L PCC7822-3 short type (24) from *Cyanothece sp.* PCC7822 and microviridin L PCC7806-4 short type (25) are shown compared to the microviridin production from pARW089-mdnA-exchange platform. All microviridins were separated by C18-RP chromatography and detected at 210 nm.

Taken together, the production of bicyclic microviridins from different precursor sequences was successfully shown, although most of them were produced in rather low concentrations.

6.9 A knockout of *mdnB* in the small microviridin expression platform

It has been observed in the fosmid system before that a knockout of *mdnB* resulted in almost no production of microviridin-like structures, although bicyclic microviridins, lacking the amide bond, were later detected to be produced in appropriate amounts from PFFARFL region mutants. Furthermore, co-expression studies with knockout fosmids and extra copies of *mdnABC* expressed from a vector never led to reactivation of microviridin

production. In contrast, addition of *mdnD* an *E* was successfully shown. At one point it was suggested, the genes *mdnABC* need to be transcribed together from one operon. To go for a more detailed analysis the small microviridin platform was used as a template to eliminate *mdnB*. Using site-directed mutagenesis a second *Aat*II restriction site was introduced at the 3´end of *mdnB* in the pARW089-mdnA-exchange platform. *MdnB* was removed by an *Aat*II digest followed by self-ligation of the plasmid. Candidates were sequenced to confirm the removal. The obtained plasmid was named pARW103-mdnBko. HPLC analysis was conducted and revealed a similar result to that obtained from the fosmid system. Traces of bicyclic microviridin were detected by mass spectrometry (data not shown).

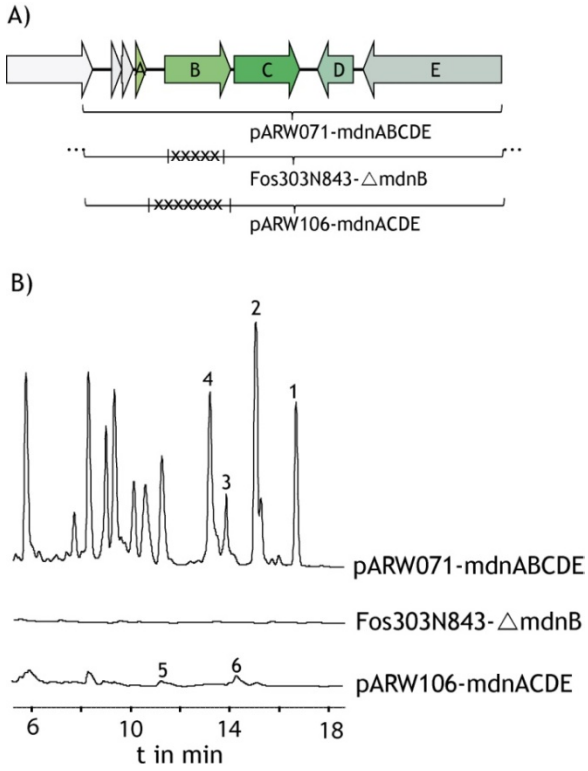

Figure 6-31: MdnB knockout in the small microviridin expression platform

A schematic representation of the small microviridin platform, carrying the genes *mdnABCDE* and the two constructs lacking *mdnB* is given in A). The dots indicate the fosmid carries about 43 kbp of Nies843 sequence. HPLC profile of microviridin production from pARW071-mdnABCDE, Fos303N843-∆mdnB and pARW106-mdnACDE is given in B). Numbers indicate the production of microviridin L (1), L1 (2), L2 (3) and L3 (4) and bicyclic microviridin L (6) and L1 (5). All microviridins were separated by C18-RP chromatography and detected at 210 nm.

Several attempts were made to knockout the ATP-dependent carboxylate–amine ligase activity of MdnB by the exchange of a highly conserved lysine (K) in the 127-AKNKLLQL-90 region of MdnB (numbers indicate omitted sequence) that is known to be involved in ATP-binding [Galperin and Koonin, 1997]. Unfortunately, mutants were never successfully constructed. It was impossible to find a functional pair of primers that could anneal and introduce the desired mutation, probably due to the high AT-content in this region. Again

co-expression of pACYCDuet-1-mdnB and pARW106-mdnACDE did not reactivate microviridin production.

7 Discussion

Attempts were made to study and manipulate a new microviridin gene cluster from the toxic cyanobacterium *Microcystis aeruginosa* Nies843.

7.1 In search of an appropriate expression system

Three different expression systems were tested for microviridin production in *E. coli*. Two of them, the fosmid and the small pDrive expression platform made use of the cyanobacterial promoter of the microviridin gene cluster, whereas in the Duet vectors the genes were expressed under the control of an *E. coli* T7 derived promoter. The use of compatible Duet vectors for microviridin expression has never been successful. Expression was only achieved when a cyanobacterial promoter was used. In terms of the efficiency factor in time, fosmids in contrast to the small pDrive based expression platform emerged to be unsuitable and ineffective for a large mutagenic approach of the microviridin gene cluster. Nevertheless, some results were obtained. First, MdnD was proven to be the acetylating enzyme in microviridin biosynthesis, which resembled results from *in vitro* studies by [Philmus, et al., 2008]. Secondly, the sequential arrangement of the genes *mdnABC* is essential for microviridin production. Thirdly, MdnE has no N- or C-terminal peptidase domain, as described for the ABC-transporter encoded as part of the cryptic microviridin gene cluster in *Nostoc sp*. PCC7120 and other bacteriocins [Havarstein, et al., 1995].

7.2 What can be learned from the transcriptional organization of the microviridin gene cluster?

Microviridin expression in *E. coli* has been shown to depend on the presence of a long untranslated leader region [Weiz, et al., 2011]. Northern hybridization showed two transcripts for *mdnA* of around 450 and 600 nucleotides in *Microcystis aeruginosa*, which both clearly exceeded the size of the coding region of *mdnA [Makower, 2010]*. The longer one could not be shown in pARW066-mdnABCDE-short. Instead a smear typical for degradation products appeared. Thus, the necessity of a longer upstream region in pARW071-mdnABCDE is assumed to have a positive effect on RNA transcript stability. It is believed this region accomodates regulatory elements [Makower, 2010]. Hints for secondary structure formation could not be found (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

Functional expression of microviridin has never been seen if *mdnA* and *B* were separated from each other. Microviridin has never been expressed from Duet vectors and

co-expression studies with *mdnB* were ineffective. Although extensively tried, the construction of a point mutant in the active center of MdnB, which would have answered many questions, failed. In their native cyanobacterial producers, the gene orthologs of *mdnABC* are often clustered together (Figure 7-1), whereas *mdnD* and *E* orthologs are either located within the gene cluster, in the close vicinity or elsewhere in the genome.

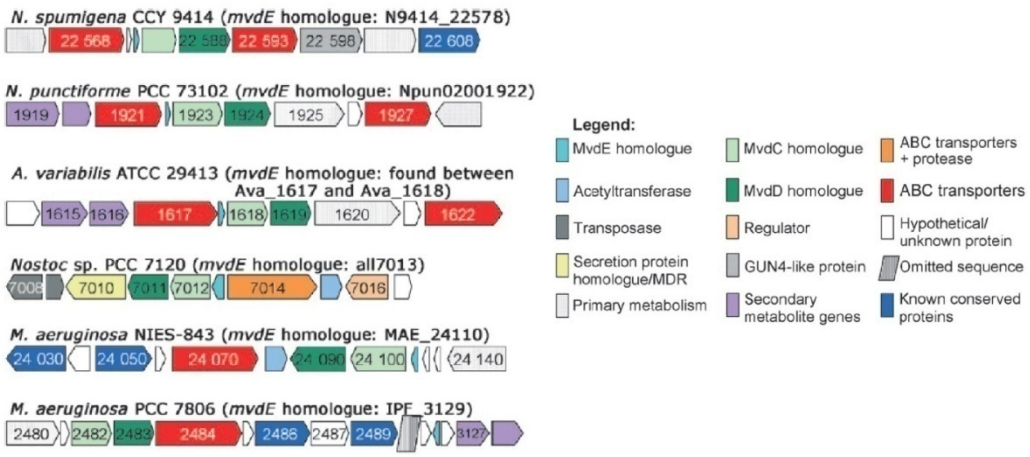

Figure 7-1: Organisation of selected putative microviridin gene clusters [Philmus, et al., 2008]

Commonly orthologs of the genes MvdE, MvdD and MvdC cluster together, whereas ABC-transporters and acetyltransferases are not necessarily located within the borders of the gene cluster. MvdE: ortholog to MdnA, MvdD: ortholog to MdnC, MvdC: ortholog to MdnB, N.: *Nostoc*, A.: *Anabaena*; M.: *Microcystis*

The related nisin gene is reported to be part of a polycistronic operon (Steen et al. 1991). A similar result was reported from investigations into the transcriptional organization of the microviridin gene cluster, conducted in parallel to this work. The genes *mdnABC* are transcribed together with the resulting polycistronic message functioning as mRNA. It seems coding sequences of *mdnA* and *B* overlap [Makower, 2010] (**Fehler! Verweisquelle konnte nicht gefunden werden.**). Such polycistronic structures of prokaryotic mRNA have profound regulatory consequences for both, transcription and translation [Kozak, 1983]. The polycistronic rule permits the translation of cotranscribed genes to be coupled closely, which enables a simple regulation and effective use of resources. MdnA would be rapidly degraded in the absence of MdnB and C. MdnD and E, located within the microviridin gene cluster, seem to be controlled by different regulatory sequences [Makower, 2010].

Thus, the use of any *in vivo* expression system that carries *mdnABC* in different backbones is inapplicable for fruitful microviridin expression using the genes of *Microcystis aeruginosa*.

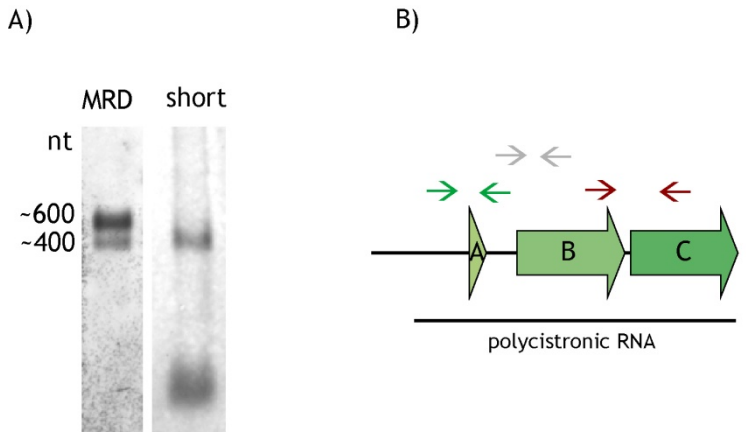

Figure 7-2: Investigations into the transcriptional organization of the microviridin gene cluster

A) Transcription of *mdnA* in microviridin J producing strain *Microcystis aeruginosa* MRD and in *E. coli* strain harboring plasmid pARW066-mdnABCDE-short with a 169 bp promoter region, nt: nucleotides; B) Schematic representation of the polycistronic operon of the microviridin gene cluster in *Microcystis aeruginosa* MRD; figure adapted from [Makower, 2010]. Arrows indicate primer positions used to map the operon. PCR on cDNA from *Microcystis aeruginosa* MRD detected products for all primer combinations and thus showed the transcription unit of the gene cluster comprises all 3 genes and a long region upstream of *mdnA*.

7.3 Analysis of the microviridin leader peptide

7.3.1 The PFFARFL motif

With the small expression platform in hand, a large mutagenic approach was started. This study represents the first detailed analysis of a microviridin leader peptide in terms of the presence of recognition motifs for the posttranslationally modifying enzymes and the protease.

A bioinformatic analysis conducted at the beginning of this mutational project disclosed that the microviridin leader peptide showed rather unusual constitutional similarities to common signal peptides in its N- to C-terminal organization, in contrast to what is known from other leader peptides [Oman and van der Donk, 2009]. Mutational analysis of the microviridin leader peptide uncovered the unique PFFARFL motif to play a key role in microviridin biosynthesis, serving as a recognition sequence for the ATP-grasp ligases (Figure 6-12). A precise collaboration of the ligases and the precursor peptide MdnA has been shown through mutational analysis of the PFFARFL region. It is well known that leader peptides of different ribosomally synthesized small peptides share some general features, for example the presence of motifs for secretion and recognition for the posttranslationally modifying or cleaving enzymes, although they are not well conserved in their primary sequence. Short sequences, such as the F(N/D)(L/V)(D/Q/E) motif of class I lantibiotics, the ELXX(B/V/I)X motif for class II lantibiotics, the FXXXL motif for microcins or the FXXXB motif for cytolysins are known to be critical for the binding of the leader to the biosynthetic enzymes, but they show some variability in their primary sequence [Müller,

2011: ; Oman and van der Donk, 2009]. In contrast, the PFFARFL region in microviridin leader peptides is highly conserved. The exchange of any individual amino acid led to profound changes in microviridin expression patterns. The activity of MdnB was immediately disabled, and thus the efficiency of microviridin production was decreased to bicyclic peptides. In contrast, point mutations introduced into other leader peptides, such as those from lacticin 481 [Patton, et al., 2008] and the microcin B17 [Cheung, et al., 2010] indicated larger plasticity, which in most cases still enabled a complete peptide processing. The exchange of two amino acids still allowed MdnC to be active, whereas mutation of three amino acids finally also abolished its activity. Thus, the PFFARFL region is of essential importance not only for MdnB, but also for MdnC. The fact that MdnC is more tolerant to changes indicates broader substrate specificity, compared to MdnB. Interestingly, bicyclic microviridins from PFFARFL mutants were produced in larger amounts compared to the tricyclic microviridins. It might be speculated that the whole amount of otherwise available ATP can go into the formation of ester bonds, which results in increased bicyclic peptide amounts.

7.3.2 The C-terminal region

The organization of the C-terminal part of the microviridin leader peptide is crucial for a certain orientation of the precursor peptide to the PFFARFL bound enzymes and thus for the correct assembly of tricyclic microviridins.

Inserting one amino acid, introduced to create an artificial cleavage site downstream of the proline in position -2 at the C-terminus of the leader, immideately decreased the level of correctly processed microviridins. Insertion of a stretch of 5 amino acids inhibited not only MdnB but severely downregulated the activity of MdnC (Figure 6-17). The dramatic effects on both ligases not only point to the need of a correct spacing between the PFFARFL and KYPSD regions for both enzymes to introduce cyclizations, but also to the basic necessity of binding to the PFFARFL region. In contrast, the introduction of an alternative C-terminal cleavage site into the nisin and haloduracin leader peptide did not hamper the modifying enzymes [McClerren, et al., 2006: ; Plat, et al., 2010].

Mutagenesis in the proline-rich region led to lowered activity of the lactam ring forming enzyme MdnB. MdnC, responsible for lactone ring formation, seemed to be nonaffected. As prolines may function as turn-inducing amino acids [Martoglio and Dobberstein, 1998], it is assumed that changes in this region interfere with the correct orientation or confirmation of the bicyclic prematured precursor peptide to the enzymes.

7.4 What role does the ABC-transporter play?

Initial experiments conducted by Ziemert and coworkers [Ziemert, et al., 2008] pointed to an accentuated need of MdnE to obtain completely processed microviridins. Thus, a more detailed analysis for the function of the ABC-transporter was set-up. Microviridin expression from the small expression platform, lacking the ABC-transporter, showed the production of only incompletely processed bicyclic microviridins. The fact that the activity of MdnB was inhibited in the absence of MdnE indicated an interaction between both enzymes (Figure 6-24). The formation of membrane-associated biosynthetic complexes has been shown numerous times for related ribosomally synthesized peptides, such as for nisin, nukacin ISK-1 and microcin B17 [Duquesne, et al., 2007: ; Nagao, et al., 2007: ; van den Berg van Saparoea, et al., 2008]. Thus, it has been speculated, the *Microcystis* prepeptide-enzyme complex requires a defined architecture provided through conformational support by the scaffolding protein MdnE.

7.4.1 Do the *in vitro* activities depend on the presence of MdnE?

In parallel to *in vivo* analysis of the microviridin gene cluster, an *in vitro* enzyme assay was intended. Initial experiments conducted in this study and by [Ziemert, 2009] involving only the proteins MdnA, B and C from *Microcystis* failed, which is now in accordance to the assumption the ABC-transporter may function as a scaffolding protein. Similarly, *in vitro* reconstitution of heterocyclization activity for microcin B17 was shown to require not only the precursor McbA, the cyclodehydrase McbB and the dehydrogenase McbC, but also McbD, that probably functions as a conformational switch [Milne, et al., 1999]. Philmus and collegues [Philmus, et al., 2008] in their experiments showed, in contrast to *Microcystis*, the orthologous *Planktothrix* microviridin machinery is able to conduct macrocyclization without additional components *in vitro*. These differences might be explained by the different C-terminal leader peptide organization in *Microcystis*. Secondary structure formation for MdnA is not given, as the peptides were synthesized. It has been shown in this work, the proline residues at the C-terminus of the leader peptide play an important role in a putative turn formation. The use of *Planktothrix* precursor and *Microcystis* enzymes and vice versa failed (Douglas Gatte-Picchi, personal communication), which indicated at that point the precursor peptide and the processing enzymes work very well synchronized. Of course, it could be tried to express MvdE in the pARW089-mdnA-exchange platform *in vivo*, to see whether it would function at all. If one would assume the lack of *in vitro* activity was at least partly associated with the absence of MdnE, it should be considered that including a single cytosolic domain has not been enough to detect *in vitro* activities. The potential dimeric architecture, described for the majority of related ABC-transporters [Rees, et al., 2009] cannot evolve from this set-up. Additionally, it cannot

be excluded further cofactors are needed. This experiment should be repeated using the complete ABC-transporter. As MdnE is a membrane protein, strategies like fusion to a maltose-binding protein or expression in micelles should be preferred. Furthermore, cell-free membrane protein expression systems are commercially available. Again it could be tried to express MdnA heterologously as a DsbC-fusion protein carrying an N- and a C-terminal tag, maybe in one of the numerous other protease deficient strains of *E. coli* designed for unstable proteins. This would help to obtain the secondary structures in the prepeptides that have been shown to be highly important.

7.4.2 Does MdnE function as a stabilizing protein?

The impact of MdnE on the stability of the putative biosynthetic complex has been analyzed using exemplarily the detection of the amount of MdnB in both, presence and absence of the transporter in *E. coli*. Higher amounts of MdnB were detected in the presence of the ABC-transporter (Figure 6-24), which indicated that the stability of the putative biosynthetic complex is increased. The fact that MdnB was only detected in the cytosol supports the assumption of MdnE, consisting of a membrane-spanning and a cytosolic ATP-binding domain, might anchor the putative complex at the inner membrane. So far, it can only be speculated that the complex is located at the inner cytosolic membrane of cyanobacteria, as they contain further membranes, like those of the thylakoid, which could also harbor the complex. A better knowledge about the function of microviridins or the localization of the transporter would help to narrow speculations. The design of an antibody against MdnE could be useful to analyze MdnE in the native host.

7.4.3 Does MdnE exhibit a function in cleavage and transport?

Beside the role of MdnE in biosynthesis, the function in processing, cleavage and transport of microviridins was analyzed. Several times ABC-transporters belonging to ribosomal biosynthetic gene clusters have been shown to exhibit a bifunctional role [Havarstein, et al., 1995]. Correct processing of microviridins has been previously shown to depend on the presence of MdnD and MdnE [Ziemert, et al., 2008]. Mutagenesis of the proline-rich region, conducted earlier, rather excluded the possibility of processing performed by a proline-specific peptidase (Figure 6-18) and supported the hypothesis the ABC-transporter might be involved. Thus, advanced studies were necessary to study the role in cleavage and transport.

All experiments, carried out in the course of this work, indicated the transporter is not involved in an ATP-dependent cleavage reaction related to transport. Under all conditions tried, the transporter is further not involved in transport, which may independently occur

from processing of microviridins, as similar amounts of microviridins were found in presence and absence of MdnE in the cytoplasm and periplasm of *E. coli* (Figure 6-22). Supernatant analysis of *E. coli* LB-cultures was impossible, due to purification problems. These results indicated, microviridins use another transport route, most likely the Sec-system, which has been also considered by bioinformatic prediction tools.

In contrast, in the cyanobacterium microviridins were exclusively found in the cytosol. Cyanobacterial culture supernatant did not contain microviridins. Studies conducted by Katharina Makower supported these results [Makower, 2010]. As *Microcystis* is hardly amenable to mutagenesis the construction of an MdnE deletion mutant was so far unsuccessful. In *E. coli* a variety of peaks for microviridin production were obtained, whereas the cyanobacterium produces only microviridin L and L1. This shows the *E. coli* processing machinery cannot manage to cleave large amounts of microviridins. It seems, the executing protease differs in the different host bacteria and is probably located outside the microviridin gene cluster. It remains to be analyzed if the protease cleaving microviridins in *E. coli* depends on the assistance of MdnE, as only in the presence of MdnE correctly processed microviridins were obtained. In consequence, *E. coli* is probably not the appropriate host to analyze cleavage and transport of microviridins. Future work should clearly go into the establishment of a cyanobacterial expression system, using a faster growing strain. It could be tried to identify the protease to be included into the *E. coli* system. All attempts to use artificial enzymatic cleavage sites directly constructed in the microviridin leader peptide failed. Searching for a completely new expression host would need a long time, as explained in this study.

The only function currently assumed and so far not reported for any ABC-transporter from a microviridin gene cluster is that of a scaffolding protein, holding the microviridin biosynthetic complex at the inner membrane. As it might be, this ABC-transporter is used to transport other and unrelated products, further studies are clearly necessary to come to a final result.

7.5 Two models for the formation of a putative microviridin maturation complex

As the presented data are the very first report about complex formation in microviridin biosynthesis, there is a need to conduct further experiments. Protein-protein interaction studies involving MdnB, C and E are necessary to understand the organization of the putative maturation complex. So far interactions were only analyzed for MdnA with MdnB and MdnC. The results are considered as preliminary data, as the synthetic precursor peptides showed some unspecific interactions. From the new insights into the mechanism

of microviridin biosynthesis and compared to what is known from literature [Duquesne, et al., 2007: ; Nagao, et al., 2007: ; Pearson, et al., 2004: ; Siegers, et al., 1996], two initial putative models for the formation of a membrane-bound maturation complex could be imagined, as shown in Figure 7-3.

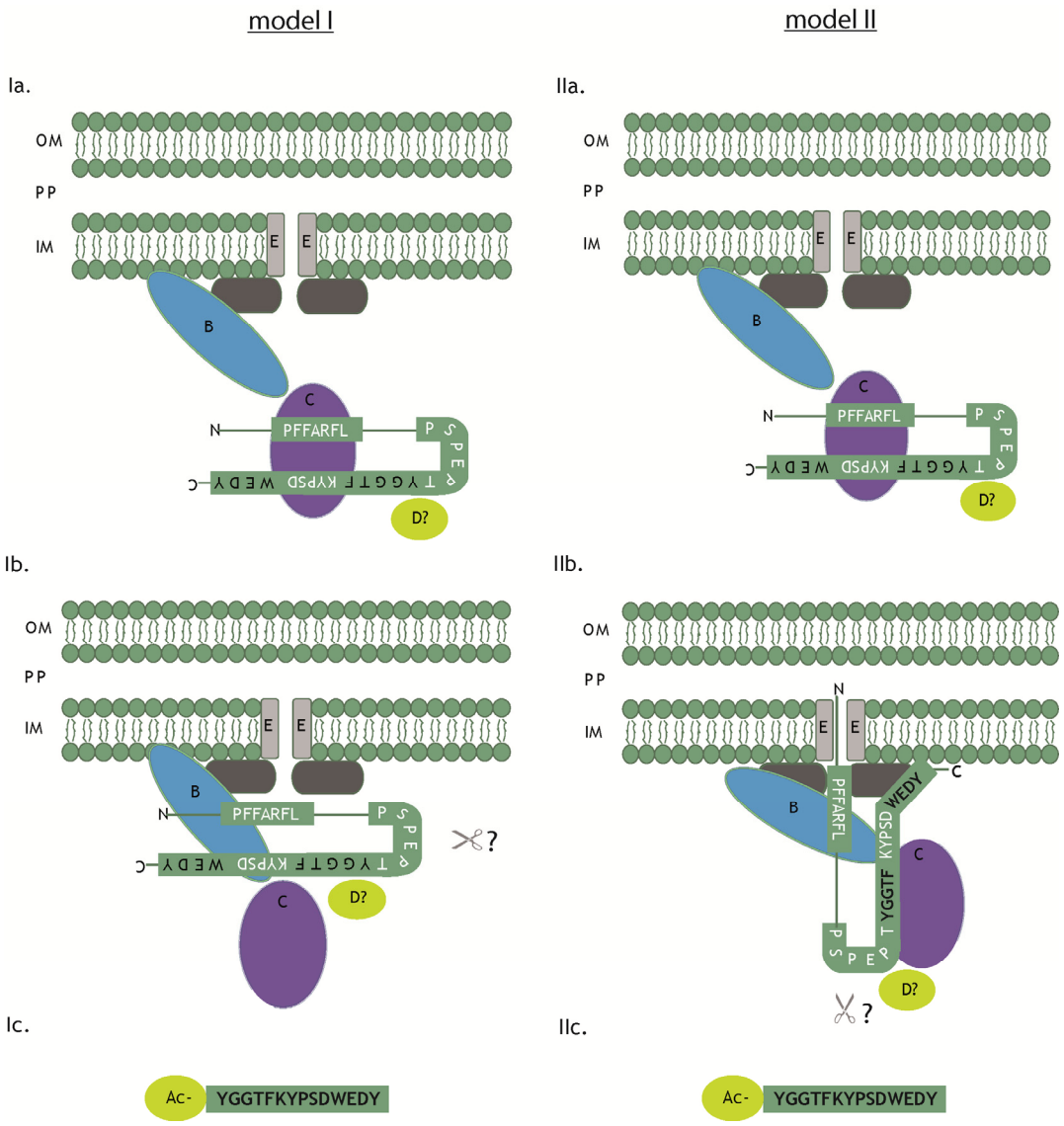

Figure 7-3: Proposed partial models for the microviridin L biosynthesis

The putative formation of an inner membrane located biosyntethic complex is shown. MdnE (E) plays a crucial role as a scaffolding protein. MdnB shown in blue (B) probably binds to MdnE. MdnC shown in purple (C) may cross-link MdnA in the cytosol. The main difference between the two models is the spatial arrangement of the prepeptide. The N-terminus of MdnA may integrate into the membrane (model II) or not (model I). The helix-breaking prolines at the C-terminus of the leader peptide introduce a turn, allowing approximation of PFFARFL and KYPSD. Once the first cyclization reaction is made, MdnC is released and the prepeptide may move closer to the membrane as the amide bond formation needs the support and presence of the transporter MdnE. Cleavage occurs by an unknown peptidase, as shown by the scissors in I and IIb. Finally MdnD, probably located within the complex, conducts acetylatation. I and IIc show the final product mdnL.

The ABC-transporter MdnE is proposed to play an essential role exhibiting putative scaffolding function and thus being the main component in complex formation. Similarly,

for most of the ABC-transporters of related peptides a dimeric channel formation is assumed or even proven [Havarstein, et al., 1995: ; Siegers, et al., 1996]. The cytosolic membrane is expected to accommodate MdnE, but the localization in another membrane system, especially in the cyanobacterium, cannot be excluded. The removal of MdnE led to an inhibition of MdnB, which might be directly stabilized by MdnE. MdnC may temporarily bind to MdnA to introduce the ester bonds. Once MdnC is released from the precursor, it is further matured by MdnB. MdnC has been shown to work in the absence of MdnB *in vitro* [Philmus, et al., 2008]. Nevertheless, MdnC worked ineffectively *in vivo* in this study, when MdnB was missing from the system (Figure 6-31). If MdnB and C interact in the putative maturation complex, the presence of MdnB is essential for complex formation. MdnB could function as an important component between MdnE and C of the putative MdnBCE complex, as MdnC can work without any difficulties in the absence of MdnE, but not in the absence of MdnB. Similar “teamwork” has been described for the proteins of the nisin biosynthetic complex [van den Berg van Saparoea, et al., 2008].

The hydrophobic PFFARFL motif, as a recognition sequence for the two ATP-grasp ligases may need to be in the close spatial vicinity to the KYPSD region that carries the amino acids involved in cyclization. The original proline-rich region in the leader peptide is indispensable, probably to induce the formation of a ß-turn that allows PFFARFL and KYPSD to come together. The formation of the proposed defined architecture within the putative membrane-associated complex, involving MdnE, B, C and the precursor peptide finally enables microviridin biosynthesis. The localization of the acetyltransferase remains so far unknown, as also the prediction tool PSORTb showed ambiguous results. Detailed studies are still outstanding, but it is assumed MdnD is part of the complex, as the possible benefit of complex formation for all enzymes might be that the precursor peptide MdnA is cyclized and processed in a well-coupled chronology, as similarly described for the nisin biosynthesis [Siegers, et al., 1996]. Processing of microviridins may occur through a cytoplasmic *E. coli* protease, which may also use MdnE as a scaffolding anchor.

In contrast to the first model, the second one shows a different hypothesis about the spatial arrangement of the precursor peptide MdnA. Maybe, the rather hydrophilic N-terminus of the precursor peptide is roped into the channel formed by MdnE to stabilize the arrangement of the precursor peptide in complex formation. The PFFARFL region with its hydrophobic character may remain outside the channel. As no transport function has been observed for the ABC-transporter, it might be this precursor organization helps the ligases to get better access to the PFFARFL region or the precursor in general to introduce macrocyclizations. Integration of a precursor peptide into the channel has been reported for nisin biosynthesis [van den Berg van Saparoea, et al., 2008]. The NisBTC complex

enzymatically dehydrates and cyclizes prenisin, but subsequently also secretes it to the periplasm for final cleavage by NisP concomitant with release of the active peptide, which has never been observed for microviridins. Compared to nisin, bacteriocins include ABC-transporters in their gene clusters that carry an N-terminal peptidase domain, located in the cytoplasm. After cytoplasmic cyclization of the small peptides, the same are cleaved and secreted by their transporters to release the active form. As microviridins were neither found in the periplasm of any cyanobacterial culture analyzed in this and other studies [Makower, 2010] nor in the culture supernatant, there is no evidence the ABC-transporter situated within the biosynthetic gene cluster translocates microviridins into the periplasm or even outside the cell. Completely processed microviridins, in contrast to bacteriocins and lantibiotics, were exclusively found in the cytoplasm in cyanobacteria. So far the only putative function for MdnE is that of a scaffolding protein. Similarly, the transporter encoded in the microcystin gene cluster, McyH, seems to function primarily as a scaffolding protein. A knockout of McyH resulted in a complete loss of microcystin production, which may cause the assumption these transporters fulfil more than only transport and cleavage function [Pearson, et al., 2004].

From the data published in literature, less facts point to the second model, for which reason the first model would be preferentially proposed. As a proof of principle the N-terminus of the leader peptide could be shortened or mutated through the exchange of hydrophilic to rather hydrophobic amino acids.

7.6 Manipulation of the microviridin core sequence

In the second part of this study, the microviridin core sequence was analyzed for its flexibility with regard to the bioactivities and stability of the resulting peptides.

The activity of original microviridin L has been estimated to be moderate against trypsin, chymotrypsin and subtilisin in comparison to the known microviridins. An inhibitory activity against elastase has not been detected up to an IC_{50}>58 µM. To conduct initial *in vivo* manipulation of the microviridin core peptide the strategy of introducing subtle changes, instead of profound manipulation has been chosen. More than once it has been reported in the past, strong manipulation and alteration involving residues participating in cyclization led to a decrease and more often even to complete loss of peptide production [Cooper, et al., 2008]. The same phenomenon has been seen for microviridin biosynthesis *in vitro* [Philmus, et al., 2009], although bioactivities of the obtained peptides were not analyzed. The choice of amino acids to be introduced by site-directed mutagenesis was mainly influenced by variants detected from the natural library during microviridin screening programs and sequence analysis.

Several changes have been successfully introduced into the KYPSDx region (KYPADx, KFPSDx, KWPSDx, KYPSDS). Microviridins were produced from most of the mutants, albeit some of them with decreased efficiency. Mutations in the PFFARFL region of the leader peptide led to bicyclic microviridins, as previously described (Figure 6-12). Thus, it is possible to generate stable bicyclic and also monocyclic variants of microviridin simply by introducing a point mutation. These data illustrate a manipulation capacity of microviridins is given, although at some points limited. Microviridin biosynthesis is a well synchronized process, involving the precursor peptide and the posttranslationally modifying enzymes. A tight co-expression and collaboration of leader and core peptide allow a constricted variability. The nisin leader peptide, which has been used in fusion with designed and novel core sequences, demonstrated a higher flexibility as ring formation and secretion for most of the medically relevant nonlantibiotic peptides has been observed [Kluskens, et al., 2005: ; Kuipers, et al., 2004].

Substitution of a residue involved in cross-linking (KYPSDx→KYPADx), led to rather inactive monocyclic peptides with decreased stability (Table 6-1). This observation has been reported from other engineered peptides as well [Cooper, et al., 2008: ; Philmus, et al., 2009]. The ligases are inflexible to changes in the crosslink-participating amino acids, as the correct ring formation serves as a stabilizer of conformations essential for bioactivity and protection against proteases of the producing strain [Bierbaum, et al., 1996].

Substitutions in the KYPSDx region using amino acids with similar characteristics that were not involved in crosslinks (KYPSDx→KWPSDx or KFPSDx) showed flexibility of the system (Figure 6-28). The efficiency of microviridin production was decreased, but the bioactivity remained unchanged or was even 2-fold increased against chymotrypsin. This position could potentially be used to engineer improved chymotrypsin inhibitors. The exchange against rather unrelated amino acids in KYPSDx→KYPSDS or KYPSDC was less successful. Serine and cysteine, for instance, carry a hydroxyl and a highly reactive thiol group, respectively. Thiol groups commonly play an important role in protein folding and other biological processes and are rather rarely occurring in a free form. It might be that the loss of production correlates with the presence of this group. Substitutions using other amino acids for this position could give a completely different result. Taken together, successful changes in the KYPSDx region have been shown, although amino acids to be introduced need to be well selected.

An amino acid exchange in position 5 (TF→TL) gave an outstanding result (Figure 6-26, Table 6-2). Several different highly potent elastase inhibiting microviridins, including microviridin B, C, G, H and I, carry a leucine in this position. It has been discussed in literature about this hypothesis earlier [Rohrlack, et al., 2003], but this study presents the

first prove for this assumption. After the exchange of TF→TL the activity against elastase increased to the nanomolar range, which is a factor of more than 100. Furthermore, chymotrypsin inhibitory activity increased about 2-fold. Thus, position number 5 is excellent to model the bioactivity of microviridins and shows great potential to study structure-function relationships. It remains open at that point, if the introduction of leucine already led to the optimum inhibitory activity that can be obtained, or not. As there are 20 proteinogenic amino acids, out of which only one has been used to show the importance of this position, there is a great potential to investigate the other residues. Furthermore, it is conceivable to introduce different functional groups or a designed side chain, if impossible *in vivo*, than it could be tried prospectively *in vitro* to generate a variety of new peptides.

The reconstructed field sample mutant of the microviridin L1 type (Ac-NVTLKYPSDWEEY) carried a leucine in position 5 and showed highly potent activity against elastases as well (Table 6-4). The initial assumption, the most abundant precursor peptide in field exhibits a beneficial bioactivity for the producing strain was confirmed. This mutant could potentially provide access to variants for structure-function relationships.

7.6.1 Expression of cryptic microviridins

Experiments, which involved a complete exchange of the precursor peptide MdnA against orthologs from other cyanobacteria, disclosed an enormous so far unutilized potential for the construction of large peptide libraries of microviridins (Figure 6-30). The expression of the closely related *Microcystis aeruginosa* PCC7806 precursor resulted in the production of large amounts of bicyclic microviridins. The diversity of *Microcystis* precursor peptides so far detected in lab strains and field samples is large and the number of precursors that still await discovery in the field is assumed to be tremendous and offers the possibility to detect novel microviridins with potentially better pharmaceutically useful characteristics. As all *Microcystis* leader peptides share high similarity in their organization, they are valueable candidates to be tested in this system. In an ongoing metagenomic project, conducted by Douglas Gatte-Picchi, several new precursor peptides were already discovered that now await their insertion into the precursor exchange platform pARW089-mdnA-exchange (Douglas Gatte-Picchi, personal communication). The expression of the precursor peptides from *Cyanothece sp.* PCC7822 has been shown. *Cyanothece sp.* PCC7822 carries about 7 different microviridin precursor-like peptides that could potentially lead to the discovery of a broad spectrum of bioactivities expressed from a single organism. Maybe another or an improved expression system for *Cyanothece* microviridins could be established in the future to isolate appropriate amounts of these microviridins.

Taken together, microviridins show high potential for bioengineering approaches, as several positions were detected to be flexible to amino acid exchanges. A mutant with a 100-fold increased inhibitory activity has been constructed. Future work is necessary to introduce more and different mutations for the design of whole libraries of highly attractive, optimized peptides to be used as anti-protease drugs or even as a proteasome inhibitor. It is believed, a systematic search and expression of so far cryptic microviridin precursors from different habitats will unravel further valueable features of these interesting compounds. An expression system for those candidates is established and ready to use.

7.7 Hypothesis about the inhibiting mechanism of microviridin

From the data obtained during this study and compared to what is known about the binding mechanism of other cyanobacterial serine protease inhibitors, such as scyptolin A from *Scytonema hofmanni* PCC7110 (Figure 7-4) or A90720A from the terrestrial cyanobacterium *Microchaete loktakensis [Lee, et al., 1994]*, some speculation about the interaction of microviridin with the serine proteases can be drawn. A report about the crystal structure of an elastase—scyptolin A complex revealed, scyptolin A uses the 4 N-terminal amino acid residues to bind specifically, but not covalently to the target at subsites S1 to S4, by forming main chain hydrogen bonds like in an antiparallel ß-sheet. The active center pocket is occupied by the macrocycle of scyptolin A and thus hydrolysis is prevented [Matern, et al., 2003].

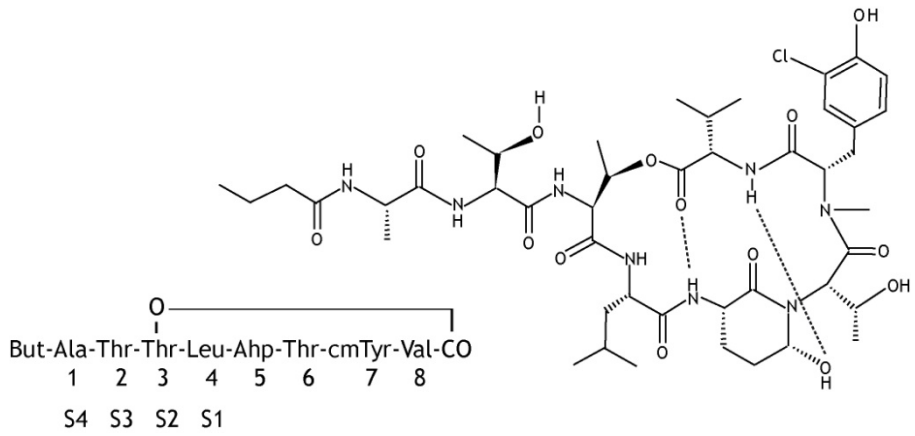

Figure 7-4: Structure of scyptolin A [Matern, et al., 2003]

The 4 N-terminal amino acids used to bind at subsites S1 through S4 of elastase are indicated. But: butyrate; Ahp: 3-amino-6-hydroxy-piperidone; cmTyr: 3`chloro-N-methyl-Tyr

Elastase consists of two ß-barrels, joined in position 126 in a sequential numbering system starting with valine at the first position and asparagine being the last amino acid in position 255. The active site residues, located between the two domains, consists of a catalytic triad with Asp108, His60 and the eponymous Ser203, the oxyanion hole and

several subsites for amino acid residues. S subsites are located C-terminally and S`subsites N-terminally of the cleavage site [Mattos, et al., 1994].

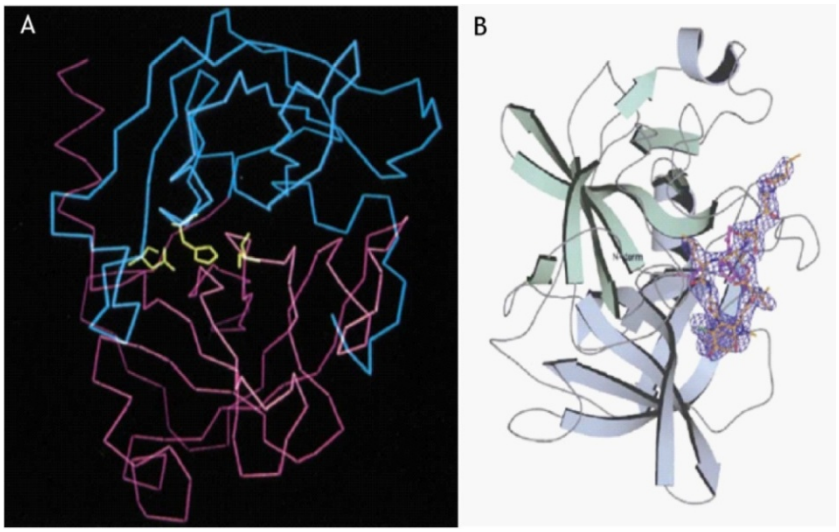

Figure 7-5: Elastase and elastase-scyptolin A inhibitor complex

A) Crystal structure of an elastase molecule. The C-terminal domain is shown in blue, the N-terminal domain in purple. The catalytic triad (Asp108, His60, Ser203, from left to right), located between the two domains, is given in yellow [Mattos, et al., 1994]; B) Stereo view of elastase-scyptolin A complex. The catalytic triad is shown beneath the surface. Scyptolin A is located in the active center pocket [Matern, et al., 2003].

As microviridins show structural and size similarities to scyptolin A, a similar binding mechanism might be assumed (Dr. Keishi Ishida, personal communication). It could be imagined, elastase interacts with microviridins in a substrate-like manner. The elastase may recognize the amino acid in position 5 of microviridins at the S1 site, whereas the remaining side chain potentially binds to the other S subsites. The macrocycle may occupy the active center pocket with the ring structure. The elastase might be capable of cleaving the peptide bond behind the amino acid in position 5, but the cyclic structure of microviridin could be saved through the potential formation of intermolecular hydrogen bonds, similar as described for scyptolin A and A90720A [Lee, et al., 1994: ; Matern, et al., 2003]. The elastase inhibitory activity of microviridins was analyzed using porcine pancreas elastase, which preferentially cleaves behind leucine, methionine and phenylalanine and resembles the attractive drug target human neutrophile elastase. Aberrant expression of this type of elastase can cause emphysema or emphysematous changes that finally lead to a disorganization of the lung structure and a breakdown of all functions. Position 5 being filled with a leucine has been shown to be more efficient compared to a phenylalanine. A methionine has not been tried, but the precursor peptide of microviridin D carries a methionine in position 5 (Figure 1-9, Table 1-1) and elastase inhibitory activity has been detected, although less efficient compared to the leucine type microviridin. In general, different amino acids in this position could be introduced to create the favorable

recognition site for the different types of elastases. Neutrophile elastase for example preferentially recognizes the small amino acids valine and alanine (http://www.uniprot.org/uniprot/P08246), which could be tried to be introduced in this position. The C-terminal part of the microviridins, including the last three amino acids, did not show any relevance for the inhibitory activity, whereas the composition of the N-terminal part was important for elastase and also chymotrypsin inhibition. The shorter the N-terminal side chain is the higher is the loss of bioactivity against elastase (Figure 7-6). The loss of the amide bond, as well as further mutations in position 7 did not significantly influence the inhibitory profile of microviridins towards the tested serine proteases.

As a proof of this binding hypothesis, a monocyclic mutant with the sequence Ac-(Y)GGTLKYPSDW should be constructed and analyzed for its elastase inhibitory activity. In case this one could keep activity, the binding mechanism would be shown to be similar to scyptolin A (Dr. Keishi Ishida, personal communication).

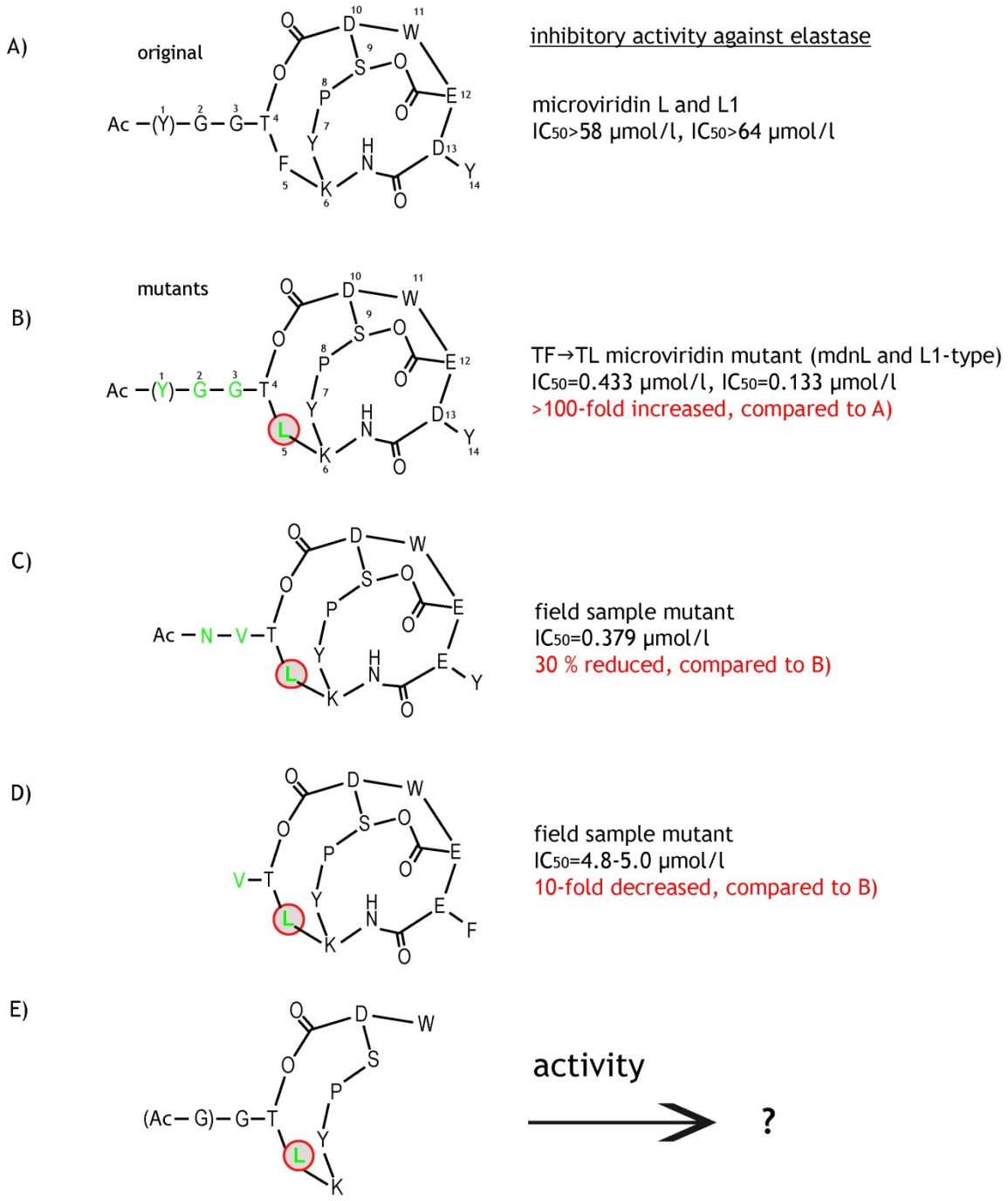

Figure 7-6: The importance of the N-terminal amino acid composition for the elastase inhibitory activity of microviridins (Dr. Keishi Ishida, personal communication)

A) Original microviridin L with its inhibitory activity against elastase. B) The TF→TL mutant of microviridin L shows about a 100-fold increased inhibitory activity against elastase, compared to original microviridin L. C) and D) represent N-terminally manipulated and shortened microviridins with gradually decreased elastase inhibitory activity. E) shows one possibility to be analyzed, if microviridin and scyptolin A have a similar inhibitory mechanism. Ac: acetylation

In comparison to other inhibitors of porcine pancreas elastase that are kept in the Brenda data base (on November 1th, 2011), the new microviridin (Ac-GGTLKYPSDWEDY) beside lyngbyastatin 7 (IC_{50}=8.3±5.4 nmol/l) [Taori, et al., 2007], isolated from the marine cyanobacterium *Lyngbya spp.*, is one of the most potent peptides for elastase inhibition

(IC_{50}=133 nmol/l). Microviridin B, which is not included in the data base, also reaches the nanomolar range (IC_{50}=26 nmol/l) [Okino, et al., 1995].

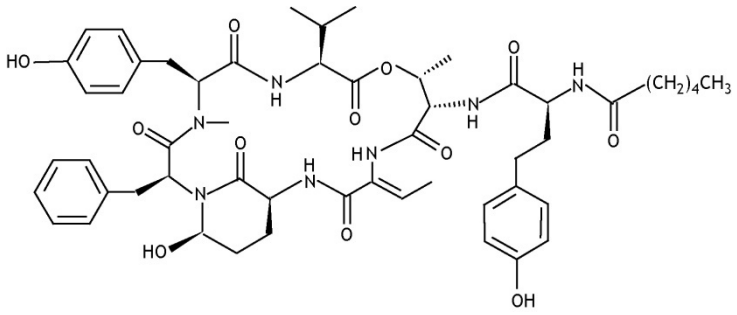

Figure 7-7: Structure of lyngbyastatin 7 [Taori, et al., 2007]

Furthermore, microviridin shows moderate inhibitory activity against trypsin, chymotrypsin and subtilisin, which are serine proteases as well. All conducted steps towards engineering microviridins did not show significant effects on the inhibitory profile against these proteases. Probably other amino acids or positions than those used and analyzed during this study are important. Subtilisin inhibition seems to depend on the presence of both ester bonds, as monocyclic microviridins lost the ability to inhibit subtilisin. Some amino acid exchanges showed a marginal increase of inhibitory activity against chymotrypsin, including those in position 5 and 7, as well as in position 9, which led to monocyclic microviridins, and thus uncovered the fact that several positions in the molecule could be analyzed and further optimized. The length of the N-terminus of the peptide seemed to be important for both. The trypsin inhibitory activity has not been modified by any of the conducted mutations. It remains questionable, if microviridin might be optimized as a trypsin inhibitor. Further studies involving substitutions in different positions using more and other amino acids are necessary to get a final result.

It could be speculated the binding mechanism of microviridin to chymotrypsin or subtilisin is not much different from the one proposed for elastase. In contrast, the serum glycoprotein alpha-1-antitrypsin, which is a well-known inhibitor of the neutrophile elastase in the lung, irreversibly binds to the serine protease by forming a covalent complex. The mechanism of inhibition involves a profound conformational change [Huntington, et al., 2000].

7.8 The potential of microviridin

7.8.1 *In vitro* synthesis as a future prospect

In vitro synthesis of ribosomal peptides is a forward looking trend in biology. Several successful reports about partial characterization of enzymatic processing of lantibiotic

precursor peptides are known [McClerren, et al., 2006: ; Müller, et al., 2010: ; Xie, et al., 2004]. Problems encountered by conventional *in vivo* biosynthesis, including poor growth of the expression host, toxic side effects, as well as proteolytic degradation of the peptide of interest could thus easily be circumvented. One of the main advantages of ribosomal biosynthesis is the possibility to custom-synthesize precursor peptides, even with expanded chemical diversity by introduction of nonproteinogenic amino acids, as it has been reported the first time in an *in vitro* mutasynthetic approach [Levengood, et al., 2009]. While this technology used to be very expensive, improved methods in peptide chemistry have made chemical synthesis more available and less expensive in recent years. To manufacture short sequences is significantly more economical than for longer peptides. Microviridin precursor peptides are relatively long, which is why heterologous expression in *E. coli* is still a competing technique. Philmus and coworkers showed MvdC and MvdD, which are orthologs of MdnB and MdnC, exhibit low *in vitro* activity [Philmus, et al., 2008]. Similar experiments conducted in this study and by Douglas Gatte-Picchi, using the same enzymes and precursor peptide (University of Potsdam, personal communication) rather pointed to inactive enzymes. So far the complex biosynthetic process of microviridin production has not proven amenable to *in vitro* reconstitution, which is why future work is necessary.

The ester bond forming ATP-grasp ligase MdnC is a unique enzyme with characteristics that have never been reported from any enzyme of the other ribosomal pathways. Once the right conditions for an *in vitro* (muta)synthesis of microviridins are established, MdnC could be manipulated to recognize substrates with broader specificity and thus might provide a system to engineer peptides and proteins unrelated to microviridins.

7.8.2 The bioactive potential

Microviridins with their potent inhibitory activity against elastases and other serine type proteases are considered to be particularly suitable in the treatment of degenerative and degradative diseases like pancreatitis, arthritis, lung emphysema, psoriasis or even cancer, caused by an uncontrolled proteolysis in the absence of a natural enzyme inhibitor [Reboud-Ravaux, 2001]. These low molecular weight peptides show bioactivity in the nanomolar range, which makes them interesting drug candidates. The small size of the compound is well suited for a simple take-up. To prevent rapid urinary excretion of very small peptides, the use of PEG in conjugation with the molecule has been shown to improve the half-life [Webster, et al., 2007]. A small dosage volume of a specific drug in general minimizes severe side effects for the consumer. The bacterial origin of microviridins makes it easy to produce large amounts of peptide. Problems of aberrant

glycosilation patterns, as reported from attempts to produce recombinant human proteinase inhibitors can be excluded [Karnaukhova, et al., 2006].

In the past, extracts of *Microcystis aeruginosa* were analyzed for their cytotoxicity. Whereas the microcystin producing strains showed cytotoxicity in a mouse model, extracts of microcystin-deficient mutants lost their strong cytotoxic properties (Prof. Elke Dittmann, personal communication). Thus, it is so far rather assumed, microviridins belonging to the spectrum of secondary metabolites produced by *Microcystis* strains are noncytotoxic protease inhibitors, which is vitally important for a potential drug development for *in vivo* application. Of course this needs to be analyzed again in more detail.

Position 5 in the microviridin core sequence showed potential for the construction of therapeutic agents with improved bioactivity. Furthermore, extensive field sampling and metagenomic studies recently conducted by Douglas Gatte-Picchi (group of Prof. Dittmann, University of Potsdam), pointed to a broader spectrum of bioactivities than so far reported for microviridins. A number of detected microviridin precursor peptides could not be cloned and expressed in *E. coli*. It was assumed, these peptides may have potential antibacterial properties that interfere with *E. coli* as an expression host (Douglas Gatte-Picchi and Prof. Elke Dittmann, personal communication).

Taken together, microviridins show pharmaceutical useful characteristics to be further analyzed as potential alternative drug candidates to especially replace currently available protease or even proteasome inhibitors in the future.

It is hoped that this work will provoke future studies to complete the investigation of the microviridin biosynthetic pathway, the organization of the putative maturation complex and the potential use as a powerful drug. Such efforts could clearly expand the knowledge about the awesome repertoire of microviridins.

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Appendix

Oligonucleotides used in this study

Primer	5`→3`sequence	Application / specificity
M13 RV	AACAGCTATGACCATG	pDrive sequencing
M13(-20) FW	GTAAAACGACGGCCAGT	pDrive sequencing
FW-pCC1/pEpiFOS	GGATGTGCTGCAAGGCGATTAAGTTGG	pCC1Fos sequencing
RV-pCC1/pEpiFOS	CTCGTATGTTGTGTGGAATTGTGAGC	pCC1Fos sequencing
mdnB fw	TTGGCTGGTTTTTGGGATAG	Library screening; *mdnB*
mdnB rv	CGATCGCATTGGAAATAGGT	Library screening; *mdnB*
P82-pET Upstream Primer	ATGCGTCCGGCGTAGA	Duet-1 vector sequencing
P83-pACYCDuetUP1 Primer	GGATCTCGACGCTCTCCCT	Duet-1 vector sequencing
P84-DuetDOWN1 Primer	GATTATGCGGCCGTGTACAA	Duet-1 vector sequencing
P85-DuetUP2 Primer	TTGTACACGGCCGCATAATC	Duet-1 vector sequencing
P86-T7 Terminator Primer	GCTAGTTATTGCTCAGCGG	Duet-1 vector sequencing
P91-mdnANies298FW	ggatccGATGGCATATCCCAACGATCA	PCR, *mdnA* N298
P92-mdnANies298RV	gaattcTTAATACTCTTCCCAGTCAGAAGG	PCR, *mdnA* N298
P93-mdnBNies298RV	ggatccgATGAAAGAATCGCCTAAAGT	PCR, *mdnB* N298
P94-mdnBNies298RV	gaattcTTAGCATACTAAAAAATCAGCGA	PCR, *mdnB* N298
P95-mdnCNies298RV	catatgACCGTTTTAATTGTTACTTT	PCR, *mdnC* N298
P96-mdnCNies298RV	agatctCGTGAGTTGACGAGGACTTCAGC	PCR, *mdnC* N298
P97-mdnDNies298RV	ggatccgATGAAAGCACTGGAAAAACTGG	PCR, *mdnD* N298; PCR N843 mdn cluster with long promoter
P98-mdnDNies298RV	gaattcTCAGCAAACCCTACTTAATTTCC	PCR, *mdnD* N298
P99-mdnENies298RV	catatgCCTCAATATACTACTAAACAAGCAACG	PCR, *mdnE* N298

Primer	5`→3`sequence	application / specificity
P100-mdnENies298RV	ctcgagTATTCTCACCCATTTTAAGGTTTTTG	PCR, *mdnE* N298
P101-mdnESequFW	TTTGTTGTTCTCCGAGGGATA	N298 *mdnE* sequencing
P102-mdnESequRV	TCCCGATCAAAGATTATCTCAA	N298 *mdnE* sequencing
P103-mdnESequFW	GGCTCTTCACTGGCAACTTT	N298 *mdnE* sequencing
P104-mdnESequRV	TTTCAGGGAGAAGAGCAGGA	N298 *mdnE* sequencing
P112-N843mdnERedETFW	atgcctcaatatactactaaacaagcaacggaaaaccctgttgtgtccgaGGCCTGGTGATGATGGCGGGATCG	Fosmid mutagenesis, PCR N843 *mdnE*
P113-N843mdnERedETRV	ctatattctcacccattttaaggtttttgggtctttatcgtaacgataatTCAGAAGAACTCGTCAAGAAGGCG	Fosmid mutagenesis, PCR N843 *mdnE*
P114-N843mdnDRedETFW	atgaaagcactggaaaaactggtgacggaacgattatatttagtccccttGGCCTGGTGATGATGGCGGGATCG	Fosmid mutagenesis, PCR N843 *mdnD*
P115-N843mdnDRedETRV	tcagcaaaccctacttaatttccagaccaagacttctctcgtgcttctaTCAGAAGAACTCGTCAAGAAGGCG	Fosmid mutagenesis, PCR N843 *mdnD*
P116-N843mdnCRedETFW	ttatgagttaactaggatttcagcgatcgcctgggagataggaaaataagGGCCTGGTGATGATGGCGGGATCG	Fosmid mutagenesis, PCR N843 *mdnC*
P117-N843mdnCRedETRV	atgaccgttttaattgttacttttagccacgataatgaaagtattcctctTCAGAAGAACTCGTCAAGAAGGCG	Fosmid mutagenesis, PCR N843 *mdnC*
P118-N843mdnBRedETFW	tcaaccgaagactaaaaaatcagcgatcgcattagaaataggtaaatctaGGCCTGGTGATGATGGCGGGATCG	Fosmid mutagenesis, PCR N843 *mdnB*
P119-N843mdnBRedETRV	atgaaagaatcgcctaaagttgttttattgttgaccatagcggcgatttTCAGAAGAACTCGTCAAGAAGGCG	Fosmid mutagenesis, PCR N843 *mdnB*
P120-N843mdnARedETFW	ttaataatcttcccagtcagaagggtatttaaaggtgcccccgtaggtagGGCCTGGTGATGATGGCGGGATCG	Fosmid mutagenesis, PCR N843 *mdnA*
P121-N843mdnARedETRV	atggcatatcccaacgatcaacaaggtaaagcacttcctttctttgctcgTCAGAAGAACTCGTCAAGAAGGCG	Fosmid mutagenesis, PCR N843 *mdnA*

Primer	5`→3`sequence	Application / specificity
P122-rpsLneo FW	GGCCTGGTGATGATGGCGGGATCG	Fosmid mutagenesis, PCR rpsLneo cassette
P123-rpsLneo RV	TCAGAAGAACTCGTCAAGAAGGCG	Fosmid mutagenesis, PCR rpsLneo cassette
P126-N843mdnAManiFW	ggatccgATGGCATATCCCAACGATCA	PCR, N843 *mdnA*
P127-Nies843mdnAManiRV	gaattcTTAATAATCTTCCCAGTCAG	PCR, N843 *mdnA*
P149-mdnENies298-108bpdelRV	ctcgagAGAATAATCAGTTAGGGACTGCA	Deletional PCR, N298 *mdnE*
P150-S•Tag primer	CGAACGCCAGCACATGGACA	pET40b(+) sequencing
P151-mdnENies298-138bpdelFW	catatgAATGGACGTGCCTTTGGAGAT	Deletional PCR, N298 *mdnE*
P164-QuikchangePFARRFL1-FW	GGTAAAGCACTTCTTTTCTTTGCTCGTTTCTTGTCCG	Qc mutagenesis of N843 *mdnA* PFFARFL region (LFFARFL)
P165-QuikchangePFARRFL1_RV	CGGACAAGAAACGAGCAAAGAAAAGAAGTGCTTTACC	Qc mutagenesis of N843 *mdnA* PFFARFL region (LFFARFL)
P166-MAE24120FW	GGCCGGATTAGTGCTTATGA	PCR and sequencing N843 mdn cluster with short promoter
P167-MAE24060RV	AGGAATTCGCCCTGAGAGTC	PCR and sequencing N843 mdn cluster with short promoter
P173-MAE24140N843FW	ggatccgATGGCATATCCCAACGATCA	PCR and sequencing N843 mdn cluster with long promoter
P174-QuikchangePFARRFL-A-FW	GGTAAAGCACTTCCTTTCTTTGCTAATTTCTTGTCCGTAAGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFANFL)
P175-QuikchangePFARRFL-A_RV	GCTTACGGACAAGAAATTAGCAAAGAAAGGAAGTGCTTTACC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFANFL)
P176-QuikchangeKYPSD-B-FW	CGGGGCACCTTTAAATGGCCTTCTGACTGGGAAG	Qc mutagenesis of N843 *mdnA* KYPSD region (KWPSD)

Primer	5`→3`sequence	Application / specificity
P177-QuikchangeKYPSD-B_RV	CTTCCCAGTCAGAAGGCCATTTAAAGGTGCCCCG	Qc mutagenesis of N843 *mdnA* KYPSD region (KWPSD)
P178-QuikchangeKYPSD-C-FW	CCTACGGGGGCACCTTTAAATTCCCTTCTGACTGGGAAG	Qc mutagenesis of N843 *mdnA* KYPSD region (KFPSD)
P179-QuikchangeKYPSD-C_RV	CTTCCCAGTCAGAAGGGAATTTAAAGGTGCCCCCGTAGG	Qc mutagenesis of N843 *mdnA* KYPSD region (KFPSD)
P184-QuikchangeFreiland1-FW	CCTGAGCCTACCTACAACGTCACCCTTAAATACCCTTCTGAC	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 1. step
P185-QuikchangeFreiland1_RV	GTCAGAAGGGTATTTAAGGGTGACGTTGTAGGTAGGCTCAGG	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 1. Step
P186-QuikchangeFreiland2-FW	CCCTTCTGACTGGGAAGAATATTAAACTCAG ATCACTTGAG	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 2. step
P187-QuikchangeFreiland2_RV	CTCAAGTGATCTGAGTTTAATATTCTTCCCAGTCAGAAGGG	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 2. step
P188-QuikchangeArginsert_FW	CCTGAGCCTACCCGATACGGGGGCACCTTTAAATAC	Qc mutagenesis, construction of trypsin cleavage site in N843 *mdnA*
P189-QuikchangeArginsert_RV	GTATTTAAAGGTGCCCCCGTATCGGGTAGGCTCAGG	Qc mutagenesis, construction of trypsin cleavage site in N843 *mdnA*

Primer	5`→3`sequence	Application / specificity
P190-QuikchangeFreiland3-FW	CCTGAGCCTACCTACAACGGCACCTTTAAATACCCTTCTGAC	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 3. step
P191-QuikchangeFreiland3-RV	GTCAGAAGGGTATTTAAAGGTGCCGTTGTAGGTAGGCTCAGG	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 3. step
P194-QuikchangeFreiland5-FW	CCTGAGCCTACCTACGGGGGCACCCTTAAATACCCTTCTGAC	Qc mutagenesis in N843 *mdnA* YGGTFKYPSDWEDY vs. YGGTLKYPSDWEDY
P195-QuikchangeFreiland5-RV	GTCAGAAGGGTATTTAAGGGTGCCCCCGTAGGTAGGCTCAGG	Qc mutagenesis in N843 *mdnA* YGGTFKYPSDWEDY vs. YGGTLKYPSDWEDY
P196-QuikchangeKYPSD-D-FW	GGCACCTTTAAATACCCTTCTGACTGCGAAGATTATTAAACTC	Qc mutagenesis of N843 *mdnA* KYPSDW region (KYPSDC)
P197-QuikchangeKYPSD-D_RV	GAGTTTAATAATCTTCGCAGTCAGAAGGGTATTTAAAGGTGCC	Qc mutagenesis of N843 *mdnA* KYPSDW region (KYPSDC)
P198-QuikchangeKYPSD-E-FW	GGCACCTTTAAATACCCTTCTGACTCGGAAGATTATTAAACTC	Qc mutagenesis of N843 *mdnA* KYPSDW region (KYPSDS)
P199-QuikchangeKYPSD-E_RV	GAGTTTAATAATCTTCCGAGTCAGAAGGGTATTTAAAGGTGCC	Qc mutagenesis of N843 *mdnA* KYPSDW region (KYPSDS)
P200-QuikchangeFreiland6-FW	CCCTTCTGACTGGGAAGAATTTTAAACTCAGATCACTTGAG	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 4. step

Primer	5`→3`sequence	Application / specificity
P201-QuikchangeFreiland6-RV	CTCAAGTGATCTGAGTTTAAAATTCTTCCCAGTCAGAAGGG	Reconstruction of a microviridin precursor previously detected in field samples; Qc mutagenesis, 4. step
P204-QuikchangeAatII-FW	CCTTCTGACTGGGAAGATTATTAAGACGTCAGATCACTTGAG	Qc mutagenesis, construction of an *Aat*II restriction site arranged behind N843 *mdnA*
P205-QuikchangeAatII-RV	CTCAAGTGATCTGACGTCTTAATAATCTTCCCAGTCAGAAGG	Qc mutagenesis, construction of an *Aat*II restriction site arranged behind N843 *mdnA*
P208-QcEheIvorleader2-FW	CGAAATTACCAGAGGAATAACTGGCGCCATGGCATATCCCAAC	Qc mutagenesis, construction of an *Ehe*I restriction site arranged behind N843 *mdnA*
P209-QcEheIvorleader2-RV	GTTGGGATATGCCATGGCGCCAGTTATTCCTCTGGTAATTTCG	Qc mutagenesis, construction of an *Ehe*I restriction site arranged in front of N843 *mdnA*
P210-PCC7120mdnprec-FW	ggcgccATGCCAGAGAATAGACAAGA	PCR; PCC7120 *mdnA* ortholog
P211-PCC7120mdnprec-RV	gacgtcCTAACCAACTGGTTGATCATCGT	PCR; PCC7120 *mdnA* ortholog
P212-PCC7822allemdnprec-FW	ggcgccATGTCTAAAAACACCGGCAAGCA	PCR; PCC7822 *mdnA* ortholog
P213-PCC7822allemdnprec-RV	gacgtcTTATGAGTCTTCCCAATCCGA	PCR; PCC7822 *mdnA* ortholog
P214-PCC7822mdnprec10-FW	ggcgccATGTCTAATAACGCCGCAGCCT	PCR; PCC7822 *mdnA* ortholog
P215-PCC7822mdnprec1-RV	gacgtcTTAATAATCTTCCCAATCGGA	PCR; PCC7822 *mdnA* ortholog

Primer	5`→3`sequence	Application / specificity
P216-PCC7806mdnprec-FW	ggcgccATGAATTATCCTAATAGCGA	PCR; PCC7806 *mdnA* ortholog
P217-PCC7806mdnprec-RV	gacgtcTTAACTATCTTCCCAGTCAGA	PCR; PCC7806 *mdnA* ortholog
P218-QuikchangeHydroxyla.-FW	CCTGAGCCTACCAACGGGGGCACCTTTAAATAC	Qc mutagenesis, construction of a hydroxylamine cleavage site in N843 *mdnA*
P219-QuikchangeHydroxyla.-RV	GTATTTAAAGGTGCCCCCGTTGGTAGGCTCAGG	Qc mutagenesis, construction of a hydroxylamine cleavage site in N843 *mdnA*
P220-QuikchangePFARRFL2-FW	GGTAAAGCACTTCCTGGCTTTGCTCGTTTCTTGTCCG	Qc mutagenesis of N843 *mdnA* PFFARFL region (PGFARFL)
P221-QuikchangePFARRFL2_RV	CGGACAAGAAACGAGCAAAGCCAGGAAGTGCTTTACC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PGFARFL)
P222-QuikchangePFARRFL3-FW	GGTAAAGCACTTCCTTTCGCTGCTCGTTTCTTGTCCG	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFAARFL)
P223-QuikchangePFARRFL3_RV	CGGACAAGAAACGAGCAGCGAAAGGAAGTGCTTTACC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFAARFL)
P224-QuikchangeKYPaD-FW	GGCACCTTTAAATACCCTGCTGACTGGGAAGATTATTAAACTC	Qc mutagenesis of N843 *mdnA* KYPSD region (KYPAD)
P225-QuikchangeKYPaD_RV	GAGTTTAATAATCTTCCCAGTCAGCAGGGTATTTAAAGGTGCC	Qc mutagenesis of N843 *mdnA* KYPSD region (KYPAD)
P226 -MdnAN843sequencingPrimer-RV	GCACTTCTCAGGATCTCAACGT	Sequencing of mdnA
P227-QuikchangePFARRFL4-FW	GCACTTCCTTTCTTTCTGCGTTTCTTGTCCGTAAGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFLRFL)

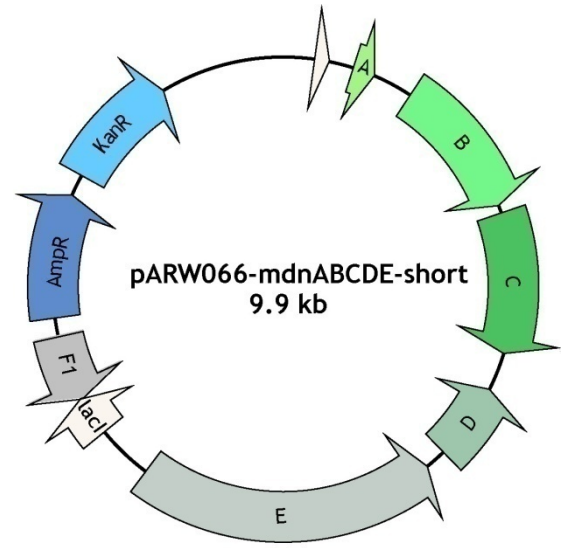
Primer	5`→3`sequence	Application / specificity
P228-QuikchangePFARRFL4_RV	GCTTACGGACAAGAAACGCAGAAAGAAAGGAAGTGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFLRFL)
P229-QuikchangePFARRFL5-FW	GCACTTCCTTTCTTTGCTCGTGCCTTGTCCGTAAGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFARAL)
P230-QuikchangePFARRFL5_RV	GCTTACGGACAAGGCACGAGCAAAGAAAGGAAGTGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFARAL)
P231-QuikchangePFARRFL6-FW	GCACTTCCTTTCTTTGCTCGTTTCGCGTCCGTAAGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFARFA)
P232-QuikchangePFARRFL6_RV	GCTTACGGACGCGAAACGAGCAAAGAAAGGAAGTGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PFFARFA)
P233-QuikchangemdnBKo-FW	GTCTTCGGTTGAGACGTCGGCAAAGCTGGC	Qc mutagenesis, construction of an *Aat*II restriction site arranged behind N843 *mdnB*
P234-QuikchangemdnBKo_RV	GCCAGCTTTGCCGACGTCTCAACCGAAGAC	Qc mutagenesis, construction of an *Aat*II restriction site arranged behind N843 *mdnB*
P235-N843mdnBAatIIKo_FW	GGTGCTTGGCAACATCATACT	Sequencing across the Qc constructed AatII restriction site behind *mdnB*
P236-QuikchangeFacXa-1-FW	CCTGAGCCTACCATCTACGGGGGCACCTTTAAATAC	Qc mutagenesis, construction of a Factor Xa Protease cleavage site arranged at the C-terminus of the N843 *mdnA* leader peptide, 1. step

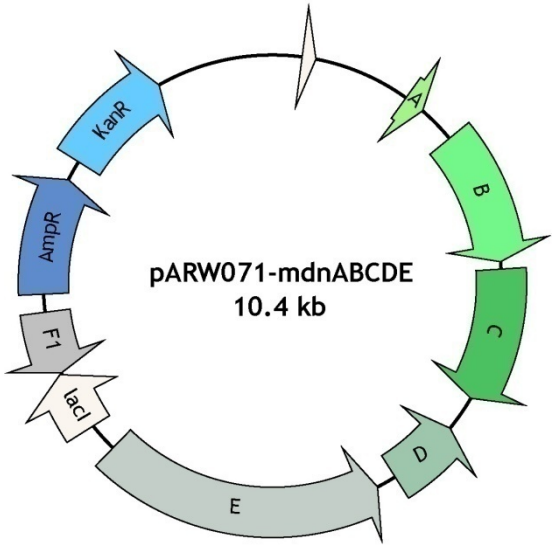
Primer	5`→3`sequence	Application / specificity
P237-QuikchangeFacXa-1_RV	GTATTTAAAGGTGCCCCCGTAGATGGTAGGCTCAGG	Qc mutagenesis, construction of a Factor Xa Protease cleavage site arranged at the C-terminus of the N843 *mdnA* leader peptide, 1. step
P238-QuikchangeFacXa-2-FW	CCTGAGCCTACCATCGAAGGCTACGGGGGCACCTTTAAATAC	Qc mutagenesis, construction of a Factor Xa Protease cleavage site arranged at the C-terminus of the N843 *mdnA* leader peptide, 2. step
P239-QuikchangeFacXa-2_RV	GTATTTAAAGGTGCCCCCGTAGCCTTCGATGGTAGGCTCAGG	Qc mutagenesis, construction of a Factor Xa Protease cleavage site arranged at the C-terminus of the N843 *mdnA* leader peptide, 2. Step
P240-QuikchangeFacXa-3-FW	GCCTACCATCGAAGGCCGAATATACGGGGGCACCTTTAAATAC	Qc mutagenesis, construction of a Factor Xa Protease cleavage site arranged at the C-terminus of the N843 *mdnA* leader peptide, 3. step
P241-QuikchangeFacXa-3_RV	GTATTTAAAGGTGCCCCCGTATATTCGGCCTTCGATGGTAGGC	Qc mutagenesis, construction of a Factor Xa Protease cleavage site arranged at the C-terminus of the N843 *mdnA* leader peptide, 3. step
P243-QuikchangemdnBaZ_FW	GAGGGCTAAAAATGGGTTATTACAACTTCGTTTGGC	Qc mutagenesis; N843 *mdnB* active center

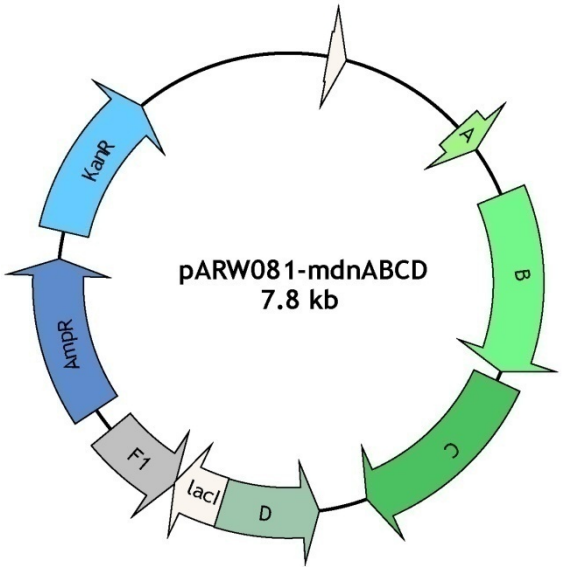
Primer	5`→3`sequence	Application / specificity
P244-QuikchangemdnBaZ_RV	GCCAAACGAAGTTGTAATAACCCATTTTTAGCCCTC	Qc mutagenesis; N843 *mdnB* active center
P245-mdnBinternsequPrimer_FW	ATCCCAGACGACTTTGGCTA	Sequencing of *mdnB* active center
P246-QuikchangemdnBaZ_FW	GAGAGGGCTAAAAATGGGTTATTACAACTTCGTTTGGCCTCAG	Qc mutagenesis; N843 *mdnB* active center
P247-QuikchangemdnBaZ_RV	CTGAGGCCAAACGAAGTTGTAATAACCCATTTTTAGCCCTCTC	Qc mutagenesis; N843 *mdnB* active center
P248-mdnBSTaqNies298FW	catatgAAAGAATCGCCTAAAGTTGT	PCR, N298 *mdnB*
P249-mdnBSTaqNies298RV	agatctcgGCATACTAAAAAATCAGCGATCG	PCR, N298 *mdnB*
P250-mdnBLys97Nies298FW	GCAAATTGAGAGGGCTAAAAATCTGTTATTACAACTTCGTTTGGCCTCAGAGTTGGCT	Qc mutagenesis; N843 *mdnB* active center
P251-mdnBLys97Nies298FW	AGCCAACTCTGAGGCCAAACGAAGTTGTAATAACAGATTTTTAGCCCTCTCAATTTGC	Qc mutagenesis; N843 *mdnB* active center
P252-QcmdnEWalkerA_FW	CCAGTGGTCGAGGAGCAAGTTCCTTGTTACGA	Qc mutagenesis; N843 *mdnE* Walker A motif
P253-QcmdnEWalkerA_RV	TCGTAACAAGGAACTTGCTCCTCGACCACTGG	Qc mutagenesis; N843 *mdnE* Walker A motif
P254-mdnEWalkerASequ_FW	CCTACAAACTCCCGATTATGA	Sequencing of Qc mutagenesis of N843 *mdnE* Walker A motif
P255-QuikchangePFARRFL7-FW	GCACTTCCTGGCTTTCTGCGTTTCTTGTCCGTAAGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PGFLRFL)
P256-QuikchangePFARRFL7_RV	GCTTACGGACAAGAAACGCAGAAAGCCAGGAAGTGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PGFLRFL)
P257-QuikchangePFARRFL8-FW	GCACTTCCTGGCTTTCTGCGTGCCTTGTCCGTAAGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PGFLRAL)

Primer	5`→3`sequence	Application / specificity
P258-QuikchangePFARRFL8_RV	GCTTACGGACAAGGCACGCAGAAAGCCAGGAAGTGC	Qc mutagenesis of N843 *mdnA* PFFARFL region (PGFLRAL)
P259-QcProlinregion1_FW	GAATCTTCCATCAAGTCT*G*CTTCC*G*CTGAGCCTACCTAC	Qc mutagenesis of N843 *mdnA* proline region PSPEPT vs. ASAEPT
P260-QcProlinregion1_RV	GTAGGTAGGCTCAGCGGAAGCAGACTTGATGGAAGATTC	Qc mutagenesis of N843 *mdnA* proline region PSPEPT vs. ASAEPT
P261-QcProlinregion2_FW	CTTCCGCTGAG*G*CTACCTACGGGGGCAC	Qc mutagenesis of N843 *mdnA* proline region PSPEPT vs. ASAEAT
P262-QcProlinregion2_RV	GTGCCCCCGTAGGTAGCCTCAGCGGAAG	Qc mutagenesis of N843 *mdnA* proline region PSPEPT vs. ASAEAT

Microviridin small expression platform vectors







Picture sources

picture	source
Figure 1-1	A) http://kentsimmons.uwinnipeg.ca/16cm05/1116/27-11x1a-Gloeothece.jpg; date: 220911 B) Annika R. Weiz C), E), F) http://www.google.de/imgres?imgurl=http://academic.reed.edu/biology/nitrogen/images/part1/nF23.big.jpg&imgrefurl=http://academic.reed.edu/biology/nitrogen/nfix1.html&usg=__s02lrZiZFFY17oAqd5R49LlaBvc=&h=274&w=288&sz=36&hl=de&start=15&zoom=1&tbnid=Lriq4aTtAvMjRM:&tbnh=163&tbnw=203&ei=NuRETszHD8rwsgapocXCBw&prev=/search%3Fq%3Dcyanobacteria%2Bgunnera%26hl%3Dde%26sa%3DG%26biw%3D1280%26bih%3D661%26gbv%3D2%26tbm%3Disch&itbs=1&iact=rc&dur=324&page=2&ndsp=15&ved=1t:429,r:6,s:15&tx=85&ty=76&biw=1280&bih=661; date: 220911 D) http://www.google.de/imgres?imgurl=http://www.botany.hawaii.edu/faculty/webb/bot311/cyanobacteria/YellowstoneBGA.jpg&imgrefurl=http://www.botany.hawaii.edu/faculty/webb/bot311/cyanobacteria/cyanobacteria.htm&usg=__jHsFJd4qQLgdJYtqxF3MUXEN84w=&h=326&w=482&sz=98&hl=de&start=11&zoom=1&itbs=1&tbnid=xPesOJ9QVYCjHM:&tbnh=87&tbnw=129&prev=/images%3Fq%3Dmarine%2Bcyanobacterium%2Blyngbia%26hl%3Dde%26gbv%3D2%26tbs%3Disch:1&ei=--k3TZ74IcOW8QODn_CfCA; date: 220911
Figure 1-2	A) http://www-cyanosite.bio.purdue.edu/images/images.html; date: 220911 B) http://toolkitbarnstable.webs.com/lovells%20beach7-16-10%20005.jpg; date: 220911

Publications

Parts of this work are already published

Weiz, A.R., Ishida, K., Makower, K., Ziemert, N., Hertweck, C., Dittmann, E. (2011), "Leader peptide and a membrane protein scaffold guide the biosynthesis of the tricyclic peptide microviridin", Chemistry & Biology 18(11): 1413-1421

Ziemert, N., Ishida, K., **Weiz. A**, Hertweck, C., Dittmann, E., "Exploiting the natural diversity of microviridin gene clusters for the discovery of noval tricyclic depsipeptides" Appl. Environ. Microbiol. 2010 Jun; 76(11):3568-74. Epub 2010 Apr 2

Publication in preparation

Weiz, A.R., Ishida, K., Gatte-Picchi, D., Ziemert, N., Hertweck, C., Dittmann, E., "Rational design of new microviridins using a minimal expression platform", in preparation

Declaration

This dissertation is the outcome of my own work. I assure I did not use any other resources than identified or referenced.

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Annika R. Weiz

2011, Humboldt University of Berlin, Germany

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